The mitochondrial protein import machinery: Role in infection, apoptosis, and organelle integrity

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Zusammenfassung

Menschliche Mitochondrien enthalten etwa 1500 bis 2000 Proteine. Die meisten dieser Proteine werden im Zellkern kodiert und im Zytoplasma synthetisiert, und müssen daher über eine spezielle Maschinerie in die Mitochondrien transportiert werden. Obwohl mittlerweile viele Details über die Wirkungsweise dieser Proteinschleusen bekannt sind, wurden einige wichtige Aspekte des Proteinimports noch nicht ausreichend untersucht. Zum einen ist nicht bekannt, ob die einzelnen Importkomplexe einen Einfluss auf die mitochondrienvermittelte Apoptose haben. Weiterhin ist offen, welche genaue Rolle der Mitochondrienimport in der Pathogenese von *Neisseria gonorrhoeae* spielt. Außerdem ist unklar, ob Faktoren des Importapparates für die Aufrechterhaltung der mitochondrialen Morphologie notwendig sind.

Um diese Fragestellungen zu untersuchen, wurden im Rahmen der vorliegenden Arbeit permanente Zelllinien hergestellt, in denen die Expression einzelner am Mitochondrienimport beteiligter Proteine mittels RNA-Interferenz (RNAi) inhibiert werden kann. Mithilfe dieser Zelllinien wurde getestet, ob die proapoptotischen Proteine Bax und Bak die Importmaschinerie benötigen, um in die äußere Mitochondrienmembran zu gelangen. Die Präsenz der beiden proapoptotischen Proteine in Mitochondrien während der Apoptose ist sehr entscheidend, da Bax und Bak in den Mitochondrien oligomerisieren und damit weitere Schritte der Apoptose einleiten. Im Widerspruch zu früheren Publikationen konnte hier gezeigt werden, dass die Translokation von Bax und Bak in die äußere Mitochondrienmembran unabhängig von Proteinimportfaktoren erfolgt. Der zweite Teil dieser Arbeit beschäftigt sich mit dem Einfluss mitochondrialer Importproteine auf die Pathogenese von *Neisseria gonorrhoeae*. Das Neisserienprotein PorB translociert während der Infektion in die Mitochondrien der Wirtszelle und induziert Apoptose. Aufgrund der strukturellen Ähnlichkeit von PorB zu bestimmten Proteinen der äußeren Mitochondrienmembran wurde bisher angenommen, dass PorB diesen endogenen Proteinen auf ihrem Importweg in die äußere Mitochondrienmembran folgt. Überraschenderweise wurde im Rahmen dieser Arbeit entdeckt, dass PorB nicht von allen Komplexen der Importmaschinerie in den Mitochondrien erkannt wird. Infolgedessen translociert es in die innere Mitochondrienmembran und wirkt dadurch toxisch auf die Wirtszelle. In einem weiteren Projekt wurde untersucht, welche Rolle die Proteinimportkomplexe der äußeren mitochondrialen Membran in der Aufrechterhaltung der Mitochondrienmorphologie spielen. Unter Verwendung der beschriebenen Zelllinien wurde entdeckt, dass in Abwesenheit des SAM (sorting and assembly) Importkomplexes die Struktur der inneren Mitochondrienmembran derangiert ist. Es wurden zudem Hinweise darauf gefunden, dass die Ursache für diesen Befund in einer Unterbrechung von Kontaktstellen zwischen den beiden Mitochondrienmembranen liegen könnte, für deren Aufrechterhaltung möglicherweise der SAM-Komplex verantwortlich ist.

Die in dieser Arbeit vorgestellten Ergebnisse erlauben neue Einblicke in verschiedene Aspekte des Proteinimports in Mitochondrien. Zudem wurde mit der Entwicklung der stabilen Zelllinien ein neues Model geschaffen, anhand dessen in Zukunft weitere Detail des mitochondrialen Proteinimports erforscht werden können.

Schlagworte: Mitochondrien, Proteinimport, Apoptose, Neisserien, Cristae.

Abstract

Human mitochondria comprise about 1500 to 2000 proteins. While only 13 proteins are encoded by the mitochondrial DNA the vast majority of mitochondrial proteins is encoded in the nucleus, synthesized in the cytosol, and translocated into mitochondria by a special protein import machinery. Although many details are now known about its function several important aspects of protein import in mitochondria were not unraveled yet. To begin with, the influence of the different mitochondrial import complexes on apoptosis is not known. Further, the exact role of the protein import machineries in mitochondria in the pathogenesis of *Neisseria gonorrhoeae* has not been clarified yet. Moreover, the question whether factors involved in protein import are required for the maintenance of the mitochondrial morphology is still unsolved.

In order to address these open issues, permanent cell lines were generated within the frame of the present thesis in which the expression of single proteins implicated in mitochondrial import can be inhibited via RNA interference (RNAi). Using these cell lines, it was investigated whether the proapoptotic proteins Bax and Bak require the import machinery in order to gain access to the outer mitochondrial membrane. The presence of both proapoptotic proteins in mitochondria is essential during apoptosis as Bax and Bak oligomerize in the outer mitochondrial membrane leading to the execution of apoptosis. In contrast to earlier publications, results presented here prove that the translocation of Bax and Bak into the outer mitochondrial membrane occurs independent of its import machineries. The second part of this thesis explores the influence of mitochondrial import proteins on the pathogenesis of *Neisseria gonorrhoeae*. The neisserial protein PorB translocates into the mitochondria of host cells during infection and induces apoptosis. Because of structural similarities of PorB to a certain class of proteins in the outer mitochondrial membrane, it was assumed that PorB would follow the import pathway of these endogenous proteins into the outer mitochondrial membrane. Surprisingly, it was found within the present study that PorB is not recognized by all complexes implicated in this import pathway. As a consequence, it translocates into the inner mitochondrial membrane to exert its toxic effect on the host cell. In a further project, the role of import complexes of the outer mitochondrial membrane in the maintenance of the mitochondrial morphology was investigated. Using the described cell lines, it was found that in the absence of the SAM (sorting and assembly) import device, the structure of the inner mitochondrial membrane was disrupted. Further, evidence was found that the reason for this phenotype could be an interruption of contact sites between the two mitochondrial membranes, whose preservation possibly requires the SAM complex.

The results presented here allow new insights into different aspects of mitochondrial protein import. Further, with the development of the stable cell lines a new model was generated that will allow future investigations on details about mitochondrial protein import.

Keywords: Mitochondria, protein import, apoptosis, neisseria, cristae.

Contents

1 Preface

The engulfment of an eubacterium by an ancestral host cell defines a milestone in the evolution of eukaryotic life, dating back between 1.5 and 2 billion years [Dyall, 04; Hackstein, 06]. Initially, this symbiotic relationship was marked by the respiration competent endosymbiont providing energy to the host. In the course of its transition to an organelle, the former endosymbiont underwent significant changes: from exclusively serving as a powerhouse, the functions of mitochondria in the cell have expanded towards playing a central role in cell metabolism and cell signaling. This transition was accompanied by the transfer of the majority of the protomitochondrial genes to the host genome, at the expense of the endosymbiont´s autonomy. The tight interlocking between mitochondria and the nucleus only became possible by the evolution of a complex import machinery enabling nuclear encoded proteins with a proper targeting signal to overcome the mitochondrial membrane barrier and reach their mitochondrial destination. Partially based on bacterial transport systems, this mitochondrial protein import machinery is well conserved among species [Dolezal, 06; Hoogenraad, 02].

By now, numerous publications unveiled the functionality of the modules of the mitochondrial import components and their interplay in great detail. Often, yeast mitochondria were used for the majority of the studies in this field, mostly due to the easy availability of temperature sensitive or conditional yeast mutants. Although very similar in principle, the protein import apparatus in mammalian mitochondria was shown to differ in some aspects from its more ancient yeast counterpart [Hoogenraad, 02; Humphries, 05; Kozjak-Pavlovic, 07]. Import studies in mammalian mitochondria were often carried out by sterical antibody blocking of import components, overexpression of dominant-negative variants, or siRNA-mediated deletions of the protein of interest. These methods, however, are either very cost-intensive, fault-prone or both. In order to overcome these obstacles, a set of stable shRNA-knockdown cell lines with an inducible knockdown of mitochondrial import components was generated within the frame of the current thesis. These cell lines entail several advantages, ranging from being highly homogeneous as a clonal population to the uncomplicated expansion of cellular material without additional costs to the possibility of knocking down essential genes due to a conditional approach. Using this tool, different aspects of mitochondrial protein import were addressed. First of all, a possible implication of the mitochondrial import machinery on apoptosis was investigated. In a second project, the fate of a pathogenic β-barrel protein from *Neisseria gonorrhoeae* that targets the mitochondria of host cells upon infection was unveiled. Finally, a novel role of Sam50, a protein that mediates the insertion of β-barrel proteins in the outer mitochondrial membrane, in the maintenance of mitochondrial membrane morphology was found.

Due to the broad spectrum of scientific fields touched in this thesis, the different projects presented within this work will be treated in closed chapters with separate introduction and discussion sections. Preceding these chapters, a general introduction about mitochondria and the mitochondrial protein import machinery is given. A final chapter summarizes the results and provides an outlook on future perspectives of the different projects.

2 Introduction

2.1 The origin of mitochondria

The architecture of mitochondria with its matrix and intermembrane space (IMS) compartments separated by an outer (OMM) and inner (IMM) membrane, folded to increase its surface, has been known for a long time. One of the first to describe mitochondria was the physician Carl Benda, who in 1898 named the organelle after the greek words mitos, meaning thread, and khondrion, grain [Benda, 98]. The choice of name reflects two major morphological features of mitochondria; their continuous threadlike reticulum that forms a three-dimensional branching structure within the cell, and their grain-like shape, as seen in electron microscopy (Figure 2-1). This resemblance with bacteria might have given first hints about the bacterial roots of mitochondria. The endosymbiont theory was published in 1967, after decades of rejection, by Lynn Margulis, although this theory was postulated already 90 years earlier by Andreas Franz Wilhelm Schimper and Konstantin Mereschkowski. Margulis´ work is based on the assumption that "an aerobic prokaryotic microbe was ingested into the cytoplasm of a heterotrophic anaerobe", resulting in the evolution of "the first aerobic amitotic amoeboid organism" [Sagan, 67].

More recent work on the mitochondrial genome suggests a close relationship of the former endosymbiont to today´s α-proteobacteria, a group of obligate intracellular pathogens. Sequence comparison revealed that the genome of *Rickettsia prowazekii*, an α-proteobacterium that causes epidemic louse-born typhus, is the nearest living relative of modern mitochondria [Gray, 99]. Uncertainty still exists regarding the host organism. One model suggests it to be a primitive amitochondriate eukaryote that already had encompartmentalized its DNA in an early nucleus [Baldauf, 03]. This assumption is based on the finding of amitochondrial eukaryotes like *Giardia lamblia*. The presence of mitochondrial heat shock protein genes in *G. lamblia*, however, suggests that this organism once

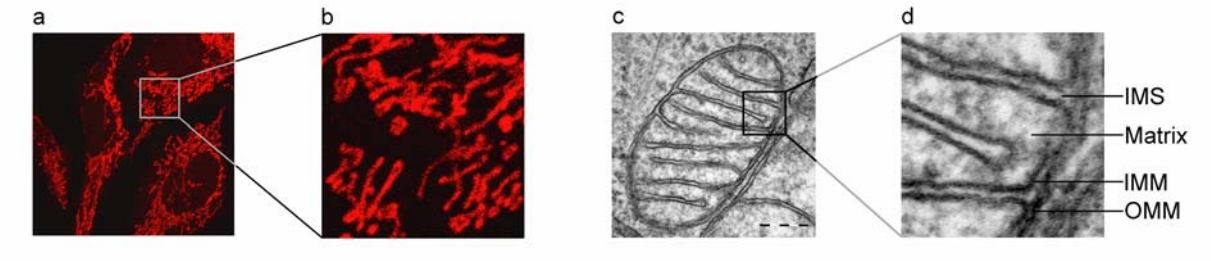

Figure 2-1. Structural features of mitochondria. (a) Mitochondria from HeLa cells stained with the mitochondria-specific dye MitoTracker Orange reveal a network organization in confocal fluorescence microscopy. (b) Enlargement of a section from a. (c) When the same cells are analyzed by transmission electron microscopy, the typical grain structure of mitochondria can be seen; scale bar, 200 nm. (d) Enlargement of a section from c. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane.

possessed a mitochondrion-related organelle, which was degraded to the genome-less mitosome [Roger, 98; Scheffler, 01]. More likely, the anaerobe organism hosting the proteobacterium was related to archaebacteria. The fact that most eukaryotic genes for replication, transcription, and translation are related to archaebacterial homologues supports this theory [Burger, 03; Embley, 06]. The most bacteria-like mitochondria were found in the protozoan *Reclinomonas americana.* Sequencing of the mitochondrial DNA (mtDNA) of *R. americana* revealed that it incorporates every gene found in mitochondrial genomes sequenced so far. The mitochondrion of *R. americana*, sometimes called "the mitochondrion that time forgot", is considered as a strong hint that all mitochondria originate from one single endosymbiotic event [Gray, 99; Scheffler, 01].

The endosymbiont´s genome, during its transition to mitochondria, was greatly reduced. Most genes migrated to the nuclear genome, probably first as duplications, to get lost later from the mtDNA once an appropriate protein transport system into mitochondria had evolved [Dyall, 04]. Human mtDNA specifies 13 respiratory chain proteins, 2 rRNAs and a set of 22 transfer RNAs (tRNA) sufficient for the translation of all mitochondrial codons [Gray, 99].

The transfer of mitochondrial genes to the nucleus that was enabled once the mitochondrial protein import machinery had been established is thought to be advantageous because it facilitates coordinate expression, enables homologous recombination, and reduces the risk of DNA damage by reactive oxygen species (ROS). Yet, the question why mitochondria retained their own genome remains unsolved, though different models exist [Allen, 03; Dyall, 04; Woodson, 08]. The "frozen accident hypothesis" proposes that there was only a certain time window where nuclear gene transfer was possible and that the process was not yet completed when the window closed. Another theory states that mitochondria can rapidly react to alterations in their redox potential by expressing respiratory chain proteins directly at the site where they are needed. The only experimental based explanation suggests that the mitochondrially encoded proteins are extremely hydrophobic, making them unsuited for the translocation machinery. One case of more recent gene doubling of a mitochondrial protein to the nucleus was found where the nuclear form of the protein decreased its hydrophobicity, enabling its import into mitochondria [Daley, 02; Dyall, 04].

An inevitable process during the migration of mitochondrial genes to the nuclear genome that accompanied the evolution of mitochondria was the development of a protein translocation machinery. Some signals that target proteins to modern mitochondria are likely to be derived from the ancestral proteobacterium [Lucattini, 04]. They might have been used for the initial targeting of proteins to a simple, bacterially derived import translocon, thereby allowing for the loss of mitochondrial genes that had already been doubled and transferred to the nucleus. Through genetic rearrangement, this targeting sequence could have been added to other genes in the nucleus, both mitochondrial and non-mitochondrial, so that the functions of mitochondria were extended further while at the same time a very intricate import machinery evolved [Kadowaki, 96; Lister, 06; Lucattini, 04].

2.2 Protein import into mitochondria

Computational studies together with proteomic approaches estimate the human mitochondrial genome to consist of 1500 to 2000 proteins [McDonald, 03; Taylor, 03; Zhang, 08]. As only 13 proteins are encoded by the human mitochondrial DNA, the vast majority of mitochondrially localized proteins are synthesized at cytosolic ribosomes and need to traverse one or two membranes to reach their place of action. Four main protein import machineries have been identified so far in mitochondria [Bolender, 08; Perry; 08; Neupert 07] (Figure 2-2). The first to be passed by virtually all mitochondrial proteins is the TOM (translocase of the outer mitochondrial membrane) complex, located in the OMM. Depending on their targeting information, proteins are subsequently sorted to their submitochondrial destination. β-barrel proteins, relicts from the bacterial ancestor of mitochondria, are

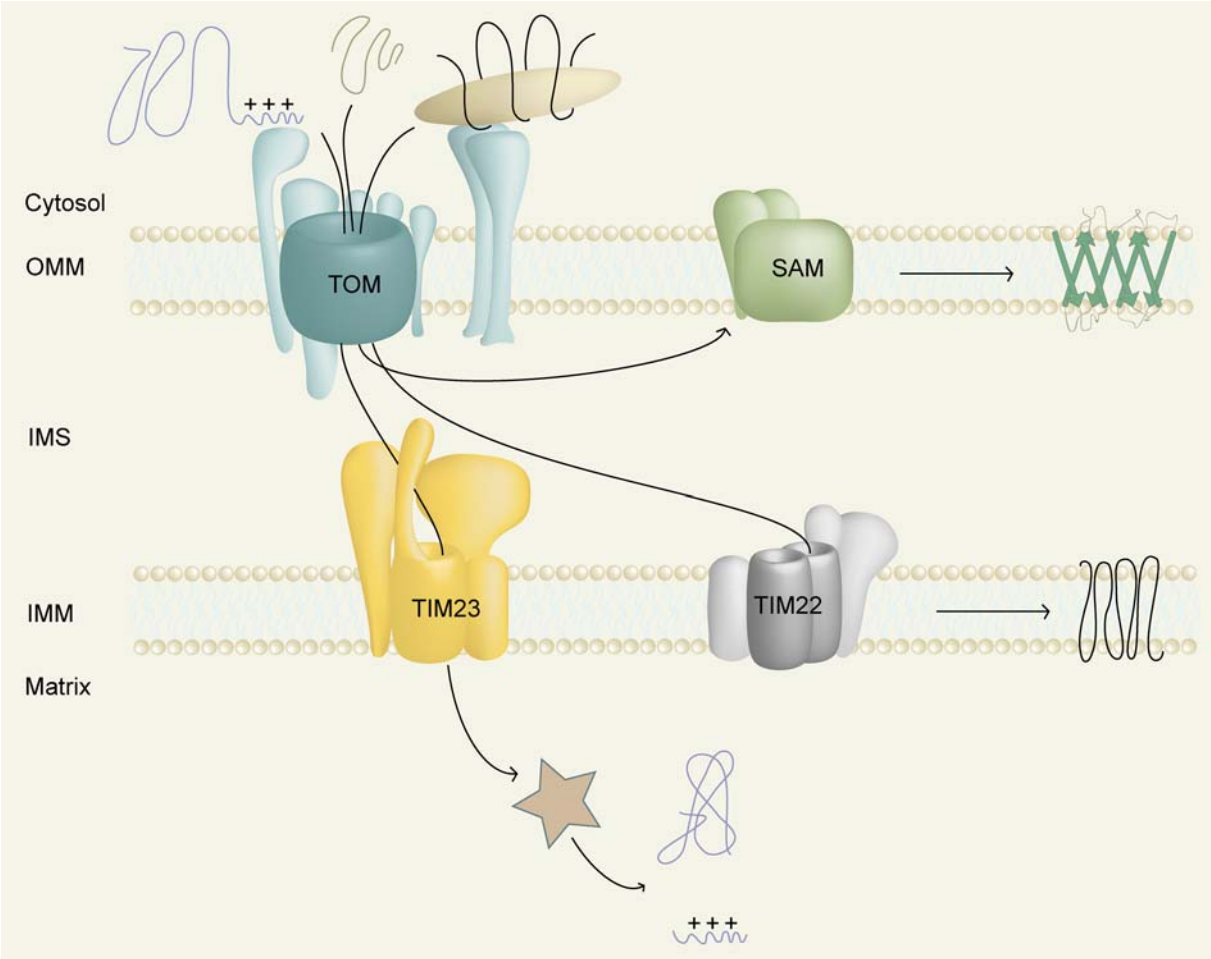

Figure 2-2. Schematic representation of the main protein machineries in mitochondria. First, precursor proteins are passed through the TOM complex. Depending on their targeting information, they are guided into the OMM by the SAM complex, into the matrix or IMS by the TIM23 or into the IMM by the TIM22 translocase. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; SAM, sorting and assembly machinery; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane.

imported and assembled into the OMM by the SAM (sorting and assembly machinery) complex. The TIM (translocase of the inner mitochondrial membrane) 22 and 23 machineries mediate transport of mitochondrial precursors to the mitochondrial IMS, the inner membrane, and the matrix. Although additional modules developed in different organisms over time, at least the core unit of each of these machineries is common to mitochondria of all eukaryotic species. This speaks in favor of one single common eubacterial endosymbiont in which the principles of protein import evolved [Dolezal, 06]. As protein import is best characterized for yeast mitochondria, it will be described first, followed by a discussion of protein import in mammalian mitochondria.

2.3 Targeting and sorting signals of mitochondrial proteins

Although cotranslational import of mitochondrial precursors has been described [Karniely, 06; Marc, 02; Suissa, 82], most mitochondrial proteins are synthesized in the cytosol and translocated into the organelle in a posttranslational manner [Endo, 03; Pfanner, 04]. Cytosolic factors keep the precursors in an unfolded state and assist in their recognition by receptor proteins at the mitochondrial surface. Due to the widely usage of mammalian transcription and translation systems, practically all of these factors were identified in mammals. The cytosolic forms of the molecular chaperones Hsp70 and Hsp90 sequester unfolded precursors and mediate the contact with the import receptor Tom70 [Fan, 06; Terada, 95; Young, 03]. The mitochondrial import stimulation factor (MSF) was found to unfold precursor proteins and improve their mitochondrial import *in vitro* [Hachiya, 93]; it could therefore be seen as a cytosolic chaperone. Similar findings were reported for the presequence binding factor (PBF) [Murakami, 90], though no further reports have been published on both MSF and PBF by other groups so far.

Diverse signal sequences drive the targeting and import of precursors to their submitochondrial destination. Most mitochondrial matrix proteins are equipped with a typical amino (N)-terminal presequence of 10 up to 80 amino acids in length. Although presequence-containing precursors do not have a common conserved targeting sequence, they do share some biochemical characteristics: they are rich in positively charged, hydrophobic, and hydroxylated amino acids and have the potential to form amphipathic α-helices [Roise, 88; von Heijne, 86]. The information contained within this presequence is sufficient to artificially target various cytosolic proteins to the mitochondrial matrix [Horwich, 85; Hurt, 84]. Among fungi and higher eukaryotes, this presequence is widely conserved [Neupert, 07]. Upon translocation of the precursor proteins into the matrix, the presequences are usually cleaved off by the highly conserved mitochondrial processing peptidase (MMP) [Gakh, 02; Mori, 80; Ou, 89], and, in the case of proteins involved in oxidative phosphorylation, additionally by the mitochondrial intermediate peptidase (MIP) [Chew, 97; Kalousek, 92].

Additional sorting sequences can lead to the targeting of presequence-containing precursor proteins to the mitochondrial inner membrane [Beasley, 93; Bomer, 97]. Thereby, precursors are pulled into the Tim23 pore; but while the presequence is chopped off by the MPP in the matrix, the peptide in transit is arrested in the IMM by a hydrophobic stretch, adjacent to the amphipathic presequence. The hydrophobic stretch acts as a so-called “stop-transfer” signal, and the precursors are

laterally released into the lipid phase [Gartner, 95; Gasser, 82; Glick, 92; Hahne, 94]. Some IMS proteins follow the same pathway, but are released into the IMS by a conserved inner membrane peptidase (IMP) [Nunnari, 93; Schneider, 91].

In approximately 30 % of all mitochondrially targeted proteins, no N-terminal presequence can be allocated [Stojanovski, 03]. Instead, these proteins contain cryptic internal import signals. This diverse group includes the multiple membrane spanning metabolite transporters of the IMM and all outer membrane proteins. Although no general sequence can be assigned to internal signals, some trends can be found in the subgroups of these proteins. The carrier proteins of the IMM contain three to six internal targeting sequences of about ten amino acids each, distributed throughout the entire protein [Brix, 99; Diekert, 99; Endres, 99; Wiedemann, 01]. In contrast to N-terminal presequences, the single targeting segments only show moderate affinity to the receptor proteins of the TOM complex. Instead, they cooperate by recruiting receptors to the precursor [Brix, 00; Wiedemann, 01].

In the outer mitochondrial membrane, proteins can be classified into signal-anchored, tail-anchored, and β-barrel proteins [Rapaport, 03]. For example, the signal-anchored outer membrane proteins Tom70 and Tom20 and tail-anchored proteins like Tom22 and Tom5 contain an N-terminal or carboxy (C)-terminal domain respectively, responsible both for targeting and insertion of the precursor. This domain is characterized by moderate hydrophobicity and, sometimes, positively charged flanking amino acids [Kanaji, 00; Suzuki, 00]. In β-barrel proteins the targeting information is encoded within the secondary structure comprised of several regions within the protein sequence [Court DA, 96; Hamajima, 88; Kutik, 08].

2.3.1 The TOM complex

Considering that so many different signals can target a protein to mitochondria, it seems astonishing that the import of nearly all mitochondrially destined proteins starts at the same import device, the TOM complex (Figure 2-3). This elaborate machinery recognizes precursor proteins by specific receptors and transfers them across the outer mitochondrial membrane.

The core of the TOM complex consists of the integral membrane proteins Tom40, Tom22, Tom5, Tom6 and Tom7. Loosely attached to this TOM core complex are the integral membrane proteins Tom20 and Tom70 [Dekker, 96; Dekker, 98; Kunkele, 98; Meisinger, 01]. According to structural predictions, Tom40 predominantly consists of β-sheets that form a cation-selective channel in the OMM with a diameter of 22 to 26 Å, most likely representing the protein conducting pore [Ahting, 99; Becker, 05; Hill, 98; Kunkele, 98; Schwartz, 99]. One TOM complex comprises two or - when Tom20 is attached to the core complex - three pores [Ahting, 99; Kunkele, 98; Model, 02; Model, 08]. Although the size of the Tom40 pore requires an unfolded precursor conformation, loopwise translocation seems to occur in the case of inner membrane carriers [Schwartz, 99; Wiedemann, 01]. Moreover, Tom40 is not a passive pore, but rather actively participates in import as its inner channel region and IMS portion reveal affinity to unfolded precursors and can prevent protein aggregation. In this way, Tom40 might help the forward movement of precursors or act as a molecular chaperone [Esaki, 03; Gabriel, 03; Meisinger, 01].

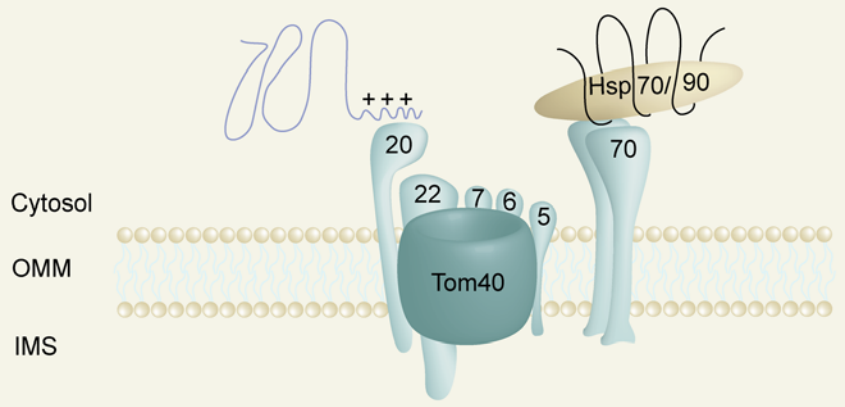

Figure 2-3. The mitochondrial TOM complex. Presequence-containing precursors are recognized by the Tom20 receptor, while mitochondrial proteins with internal targeting information, often in conjunction with cytosolic chaperones like Hsp70, are primarily bound by Tom70. Precursors are passed on to the pore of the TOM complex, Tom40, over the central receptor Tom22. Tom40 mediates the transfer of precursors across the OMM. The small Tom proteins Tom5, 6 and 7 stabilize the TOM complex and assist in the import of precursors. Hsp, Heat shock protein; IMS, intermembrane space; OMM, outer mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane.

Tom20 and Tom70 are the major receptors of the TOM complex. Both are N-terminally anchored in the OMM and are equipped with large hydrophobic domains protruding into the cytoplasm. Although they differ in substrate specificity, both receptors seem to have overlapping functions and can - at least partially - substitute for each other [Brix, 97; Brix, 99]. Tom70, organized in a dimeric form, preferentially binds hydrophobic carrier proteins with multiple internal signal sequences, and, with lower affinity, also binds precursors with an N-terminal presequence [Ryan, 99; Schlossmann, 94; Wiedemann, 01; Young, 03]. The cytosolic segment of Tom70 builds a binding groove for cytosolic Hsp70 and a pocket for the association with hydrophobic precursor proteins; both sites are formed by conserved tetratricopeptide repeat (TPR) motifs [Chan, 06; Wu, 06]. Tom20 is the main receptor for precursors equipped with an N-terminal presequence [Harkness, 94; Sollner, 89]. Like Tom70, it contains a hydrophobic binding groove in its cytoplasmic domain that binds to the hydrophobic face of the presequence and TPR motifs that potentially interact with cytosolic chaperones [Abe, 00; Yano, 04]. Recently, a model was proposed where two molecules of Tom20 build the central unit of the TOM complex, surrounded by three pores of Tom40, and three peripherally associated molecules of Tom22. This setup provides a possible explanation for the crucial role of Tom20 in the assembly of the three pore complex [Model, 08]. In contrast to the other receptors, Tom22 is anchored in the OMM via its C-terminus and has a short IMS domain [Lithgow, 94]. The N-terminal, cytoplasmic domain of Tom22 reveals receptor- and chaperone-like function similar to Tom20 [Brix, 97; Brix, 99; Yano, 00; Yano, 04]. Sometimes, Tom22 is considered the major receptor receiving precursors from Tom20 and Tom70 and passing them over to Tom40 [Pfanner, 01]. In the IMS Tom22 binds to precursors and assists in the import of matrix proteins by contacting the IMM translocase TIM23 [Chacinska, 05; Moczko, 97; Mokranjac, 05]. Besides its receptor function, Tom22 is essential for the integrity of the TOM core complex and involved in gating of the Tom40 channel [Meisinger, 01; van Wilpe, 99].

The small Tom subunits are tail-anchored transmembrane proteins that are closely associated with Tom22 and Tom40 [Dembowski, 01]. The loss of individual small Tom proteins does not affect mitochondria significantly, but simultaneous depletion of all three Tom proteins is lethal [Dekker, 98; Dietmeier, 97; Sherman, 05]. Tom5 assists in the insertion of substrate into the Tom40 pore and in some cases acts as a receptor [Dietmeier, 97; Kurz, 99]. Tom6 is in close contact with Tom22 [Dembowski, 01] and is thought to assist in the core TOM complex assembly [Dekker, 98; Kassenbrock, 93; Model, 02] and the regulation of complex stability, probably in conjunction with Tom5 and Tom7 [Alconada, 95; Honlinger, 96; Schmitt, 05; Sherman, 05].

The TOM complex is highly conserved between fungi and mammals [Hoogenraad, 02] (Figure 2-7 and Table 1). Homologues of all yeast TOM proteins with similar or identical functions were identified in mammalian mitochondria. A 38 kDa protein homologous to the fungal Tom40 was found in rat [Suzuki, 00] and human mitochondria [Abdul, 00]. It exhibits a predicted β-barrel structure and accomplishes the same function as the yeast Tom40 [Humphries, 05]. Human Tom22, a protein of 16 kDa with a C-terminal IMS domain and an acidic cytoplasmic N-terminus that recognizes presequence-containing precursor proteins, is also tightly associated with the TOM core complex and can be coimmunoprecipitated together with Tom20 [Suzuki, 00; Yano, 00]. Human Tom20, also smaller than its yeast counterpart, exhibits similar features as yeast Tom20. It has a highly acidic cytosolic C-terminus, binds the hydrophobic face of N-terminal presequences and is only loosely associated with the TOM complex [Abe, 00; Goping, 95; McBride, 96; Seki, 95; Terada, 97]. Mammalian Tom70, like in yeast, is slightly attached to the TOM complex and recognizes proteins with internal targeting information [Alvarez-Dolado, 99; Suzuki, 02]. Homologues of the small Tom proteins Tom5, Tom6, and Tom7 were also identified in human mitochondria recently and, as in yeast, they seem to stabilize the TOM complex, but do not seem to play a role in protein import itself [Johnston, 02; Kato, 08]. In addition, a receptor named Tom34, identified only in mammals, shares homology to Tom70 and entails a TPR-motif found to mediate protein-protein interactions of receptors with cytosolic chaperones or preproteins [Chewawiwat, 99; Young, 98].

The driving force for protein translocation across the TOM complex is not known yet. According to the “acidic chain” hypothesis, acidic residues on cytosolic and IMS domains of TOM components could interact with positively charged presequences through electrostatic interactions in a way that preproteins are passed down an electrochemical gradient from low to increasingly higher affinity receptors [Bolliger, 95; Komiya, 98]. The Tom22 IMS domain, however, is not essential for protein import [Court, 96] Moreover, the mammalian Tom22 lacks an accumulation of acidic residues in the IMS domain [Yano, 00]. A more recent model proposes that preproteins move along binding sites with an increasing affinity for structural features rather than for acidic regions [Pfanner, 01].

2.3.2 The SAM complex

β-barrel proteins are a unique species of evolutionarily conserved membrane proteins that occur in the outer membrane of mitochondria and gram-negative bacteria. All β-barrel proteins in eukaryotes are encoded in the nucleus and are transported through the OMM by the TOM complex.

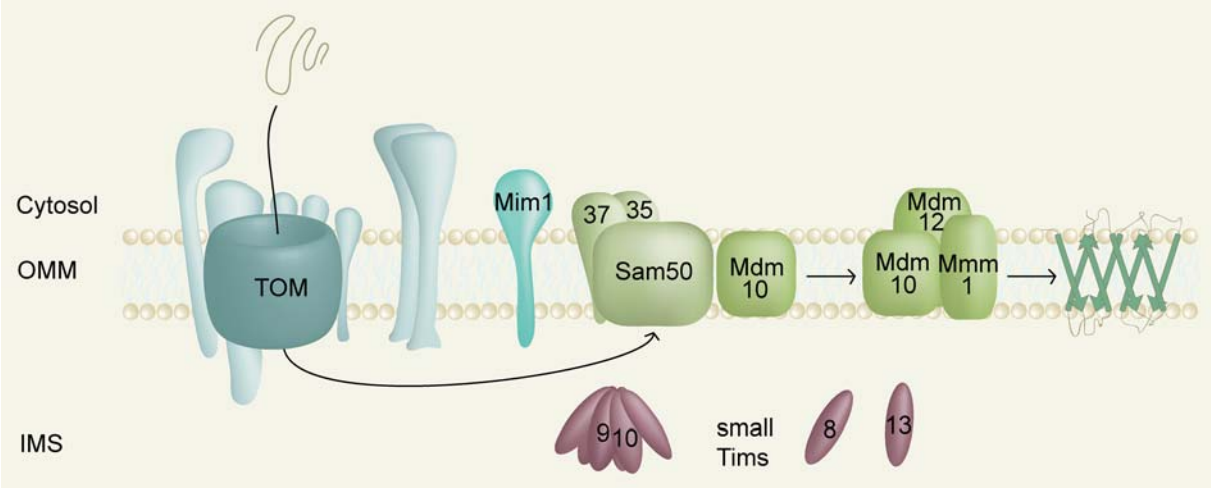

Figure 2-4. The SAM complex of yeast mitochondria. β-barrel proteins are transferred from the TOM to the SAM complex with the help of the IMS Tim chaperone complexes (small Tims). The central core, Sam50, assembles precursors in the OMM, assisted by Sam35 and Sam37. Final steps in the assembly of β-barrel proteins are carried out by the MDM complex units Mdm12 and Mmm1. Mdm10 temporarily associates with the SAM complex to build the TOM complex. Mim1 is also specialized on TOM complex assembly. IMS, intermembrane space; MDM, mitochondrial distribution and morphology; OMM, outer mitochondrial membrane; SAM, sorting and assembly machinery; TOM, translocase of the outer mitochondrial membrane.

Only few eukaryotic β-barrel proteins were identified so far, including VDAC (voltage-dependent anion-selective channel), Tom40, and Sam50. They are assembled into their mature high molecular weight complexes in the OMM by a specialized protein machinery, the SAM (sorting and assembly machinery) or TOB (topogenesis of mitochondrial outer membrane β-barrel proteins) complex (Figure 2-4). The central component of this machinery is Sam50 [Kozjak, 03] or Tob55 [Paschen, 03], of which homologues exist in all eukaryotes including plants and mammals [Gentle, 04]. Sam50 exhibits high sequence similarity to Omp85, a β-barrel protein found in the outer membrane of gram-negative bacteria [Gentle, 04; Schleiff, 05; Voulhoux, 04]. Interestingly, in *Neisseria meningitidis* Omp85 was found to assemble β-barrel proteins in the outer bacterial membrane, providing evidence for a functional homology to Sam50 besides structural parallels [Voulhoux, 04]. In yeast, the outer membrane proteins Sam35 (Tom38, Tob38) [Milenkovic, 04; Waizenegger, 04] and Sam37 (Mas37) [Gratzer, 95; Paschen, 03; Wiedemann, 03] build the SAM core complex together with Sam50 and assist in the assembly of β-barrels in the OMM in a sequential manner [Chan, 08]. The transfer of β-barrel proteins from the TOM to the SAM complex is mediated by the TIM chaperone complexes Tim8-Tim13 and Tim9-Tim10, located in the IMS [Hoppins, 04; Wiedemann, 04]. Recently, the MDM complex (mitochondrial distribution and morphology), consisting of Mdm12 and Mmm1, was shown to act downstream of the SAM complex in the assembly of β-barrel precursors [Meisinger, 07].

Specialized devices exist for the assembly of the TOM complex. Mdm10 can associate with both the MDM and the SAM complex; in conjunction with the latter, Mdm10 is involved in late steps of the TOM complex assembly [Meisinger, 04]. Mim1 forms an alternative complex with the SAM core complex and promotes integration of signal-anchored Tom proteins into the TOM complex [Becker,

08; Hulett, 08; Ishikawa, 04; Popov-Celeketic, 08; Waizenegger, 05]. Moreover, the SAM complex was recently found to be involved in the import of Tom22 [Stojanovski, 07], indicating that the SAM complex is not solely specialized in the assembly of β-barrel precursors.

β-barrel assembly in mammalian cells is far less understood (Figure 2-7 and Table 1). Human Sam50, like its fungal counterpart, is essential for the assembly of β-barrel proteins in the outer membrane of mammalian mitochondria [Gentle, 04; Humphries, 05; Kozjak-Pavlovic, 07]. Besides Sam50, only Metaxin 1 and Metaxin 2 were found to be involved in β-barrel assembly. Metaxin 1 shares sequence homology with yeast Sam37 [Armstrong, 97] and interacts with Sam50 and Metaxin 2 in β-barrel protein assembly, most likely in different protein complexes [Kozjak-Pavlovic, 07; Xie, 07]. The putative counterpart of Metaxin 2 is Sam35, although no sequence similarities can be assigned [Armstrong, 99]. Further, a gene for human Metaxin 3 was identified, but a possible membrane association and participation in protein import was not investigated yet [Adolph, 04].

2.3.3 The MIA machinery

Numerous proteins in the IMS with masses smaller than 20 kDa carry a characteristic conserved cysteine motif that is required for the binding of cofactors, for example metal ions. These proteins are equipped with an internal mitochondrial targeting information and shuttled through the TOM complex into the IMS with the help of the MIA (mitochondrial intermembrane space import and assembly) complex [Chacinska, 04; Naoe, 04]. Its central core, Mia40, contains six conserved cysteine motifs that mediate the folding of precursors and their assembly into mature complexes, and their release into the IMS through sequential formation of intramolecular disulfides bonds [Mesecke, 05; Milenkovic, 07; Sideris, 07]. Mia40 is reoxidized by the sulfhydryl oxidase Erv1 (essential for respiration and viability 1), that passes the electrons over cytochrome *c* to the respiratory chain [Allen, 05; Mesecke, 05].

A homologue of Mia40 containing the conserved cysteine residues was identified in higher eukaryotes including humans [Terziyska, 05] (Figure 2-7 and Table 1). Like in yeast mitochondria, human Mia40 also functions in the import and assembly of small IMS proteins [Chacinska, 08; Hofmann, 05]. The human homologue, however, lacks a transmembrane segment that couples its yeast counterpart to the IMM.

2.3.4 The TIM23 complex

Proteins with an N-terminal presequence that are destined for the IMM or the matrix are handed over from the TOM complex to the TIM23 translocase (Figure 2-5). The core subunit of this complex is Tim23, a channel-forming transmembrane protein with an extensive IMS domain [Truscott, 01]. In order to preserve the membrane potential across the IMM ($\Delta\psi$), the channel is tightly regulated by the TIM23 subunit Tim50 [Dekker, 93; Meinecke, 06]. Tim50 also interacts with precursors at the IMS side of the TOM complex and assists in their binding to the IMS domain of Tom22, facilitating their transfer through the OMM [Geissler, 02; Mokranjac, 03; Yamamoto, 02].

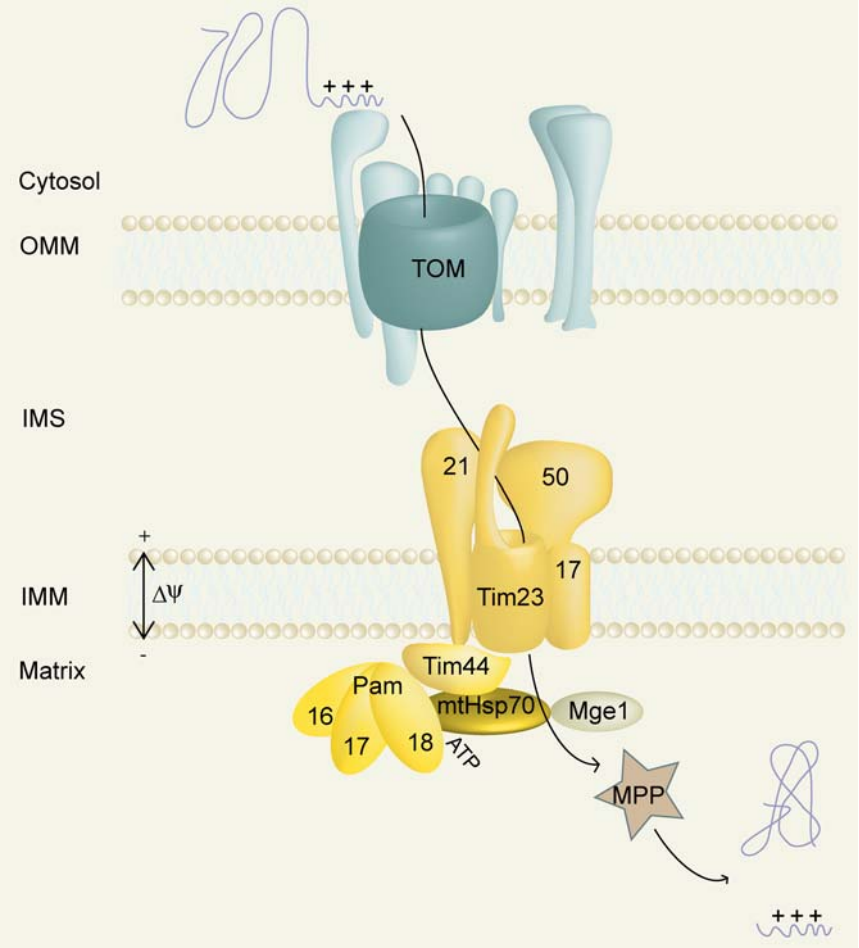

Figure 2-5. The yeast TIM23 complex. Presequence-containing precursors are shuttled through the IMM by the Tim23 pore. The accessory TIM23 factors Tim21 and Tim50 mediate the transfer of precursors from the TOM to the TIM23 complex. Tim50 also regulates the Tim23 pore gating. The core component of the PAM complex, mtHsp70, pulls precursors into the matrix in a ATP-dependent manner. Thereby, hydrolysis of ATP is assisted by Pam16, Pam17, Pam18 and nucleotide exchange factors like Mge1. The PAM complex is tethered to the TIM23 complex by Tim44. Presequences are cleaved off by the mitochondrial processing peptidase. IMM, inner mitochondrial membrane; IMS, intermembrane space; MPP, mitochondrial processing peptidase; OMM, outer mitochondrial membrane; PAM, precursor-associated motor; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane; Δψ, mitochondrial inner membrane potential.

Another factor of the TIM23 complex, Tim21, competitively binds Tom22 presumably to achieve the release of the precursor from Tom22 [Albrecht, 06; Chacinska, 03]. The precursor then interacts with the IMS domain of Tim23 and is channeled through the pore [Truscott, 01]. The driving force for the IMM translocation is Δψ, that activates the Tim23 channel and exerts an electrophoretic force on the positively charged presequences. Furthermore, the ATP-dependent PAM (precursor-associated motor) complex drives translocation through the TIM23 complex [Krayl, 07; Truscott, 01].

The core component of the PAM complex, the matrix form of Hsp70 (mtHsp70), binds precursors and pulls them into the matrix with the help of Tim44 that transiently links the PAM motor to the

TIM23 complex [Blom, 93; Hutu, 08; Schneider, 94]. Matrix import is further regulated by J-protein family members that bind and promote ATPase activity of Hsp70 members. Pam18 is a DnaJ-like protein and stimulates the ATPase activity of mtHsp70. Pam16 controls the activity of Pam18, while Pam17 is thought to help in the assembly of the Pam18-Pam16 module [D'Silva, 05; Li, 04; van der Laan, 05]. Nucleotide release factors like Mge1 or Mdj1 in the matrix assist in the exchange of ADP by ATP [Liu, 03; Schneider, 96]. The PAM complex is associated with the TIM23 translocase only in the absence of Tim21 [Chacinska, 05]. When not PAM, but Tim21 is bound to the TIM23 complex, precursors containing a stop-transfer signal following their N-terminal presequence are inserted into the channel and are laterally released into the IMM [van der Laan, 06]. The switch between Tim21 and PAM is mediated by the TIM23 complex protein Tim17 [Chacinska, 05].

Only little is known about the import of presequence-containing precursors in mammalian mitochondria, although it seems to follow a similar principle (Figure 2-7 and Table 1). Homologues of Tim23, Tim17, Tim50, Tim44 and mtHsp70 were identified in mammalian organisms [Bauer, 99b; Bomer, 96; Guo, 04; Ishihara, 98; Wada, 98], whereas Tim21-like proteins were not characterised yet, although a respective gene does exist in higher eukaryotes [Sun, 07]. Human Tim17 is encoded by two genes whose products build two different complexes with human Tim23 for yet unknown reasons [Bauer, 99b]. Human Tim50 is a 40 kDa protein that forms a complex with Tim23 and exerts phosphatase activity *in vitro* [Guo, 04]. In contrast to the membrane integrated form in yeast, human Tim44 is soluble in the matrix or only loosely associated with the IMM [Bauer, 99b; Ishihara, 98; Kronidou, 94; Wada, 98]. It associates with mtHsp70 and can be dissociated by adding ATP [Ishihara, 98]. Homologues of the Pam proteins Pam16 and Pam18 also exist in humans, though their implication in mitochondrial protein import still needs to be investigated [Davey, 06; Jubinsky, 01]. Mutations in the human Pam18 gene cause DCMA syndrome, a severe form of cardiomyopathy [Davey, 06].

2.3.5 The TIM22 complex

Carrier proteins with internal targeting information are guided to the TIM22 translocase by the soluble, hexameric chaperone complexes Tim9-Tim10 and - in the case of the Tim23 precursor - Tim8-Tim13 [Davis, 07; Vial, 02; Webb, 06] (Figure 2-6). Docking of these complexes to the TIM22 import unit is mediated by Tim12 that passes the carrier substrate to the translocase [Gebert, 08; Koehler, 98; Ryan, 99; Sirrenberg, 98], consisting of the integral membrane proteins Tim22, Tim54, and Tim18 [Kerscher, 97]; [Koehler, 00]. Tim22 is a channel-forming protein with sequence homologies to Tim23 and Tim17 [Sirrenberg, 96]. It forms a twin pore whose gating is dependent on $\Delta\psi$ across the IMM and on the binding of precursor substrate [Kovermann, 02; Peixoto, 07; Rehling, 03]. Tim54 could exert a similar function as Tim50 in regulating the pore, whereas Tim18 acts in the TIM22 complex assembly [Kerscher, 97; Wagner, 08].

Homologues of Tim22 and of the small Tim proteins (Tim8a, Tim8b, Tim9, Tim10a, Tim10b, and Tim13) have been identified in mammals [Bauer, 99b; Koehler, 99], whereas genes encoding Tim54 and Tim18-like proteins are not present in higher eukaryotes (Figure 2-7 and Table 1). A mutation in Tim8a causes the neurodegenerative disorder Mohr-Tranebjaerg syndrome [Koehler, 99].

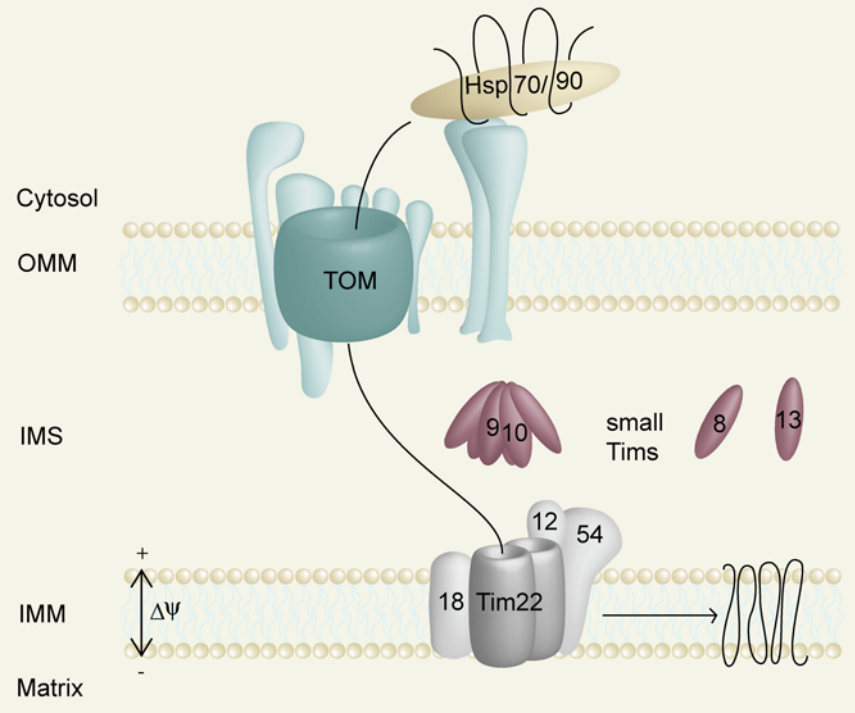

Figure 2-6. The TIM22 complex of yeast mitochondria. Inner membrane carriers are guided to the TIM22 translocase by chaperonic complexes consisting of the small Tim proteins Tim8, Tim9, Tim10 and Tim13. Docking of the chaperone complexes to the TIM22 machinery occurs via Tim12. The carrier precursors are inserted into the IMM via the central pore Tim22 in a membrane potential -dependent way. Tim18 is crucial for TIM22 complex assembly, Tim54 may regulate the pore activity. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane; Δψ, mitochondrial inner membrane potential.

2.3.6 Evolution of mitochondrial import machines

Considering the intricacy of modern mitochondrial protein import engines it seems reasonable to ask how these molecular machines have evolved. With the exception of the SAM complex, all main import machines are thought of having developed *de novo* in the common endosymbiotic ancestor of all mitochondria [Dolezal, 06; Herrmann, 03; Lister, 06].

The Omp85 protein family is conserved among gram-negative bacteria and eukaryotes [Gentle, 04; Voulhoux, 04]. Sam50-like proteins, characterized by a size of 50 kDa, a C-terminal 30 kDa domain highly conserved from bacteria to man, and the N-terminal polypeptide translocase domain (POTRA) can be found in all eukaryotes. Membrane insertion through the SAM complex taking place from the IMS side of the OMM reveals parallels to the insertion of bacterial β-barrel proteins from the periplasm. In bacteria, integration of β-barrel proteins into the outer membrane requires the chaperones Skp and SurA. The mitochondrial small Tim proteins administrate equal function and are

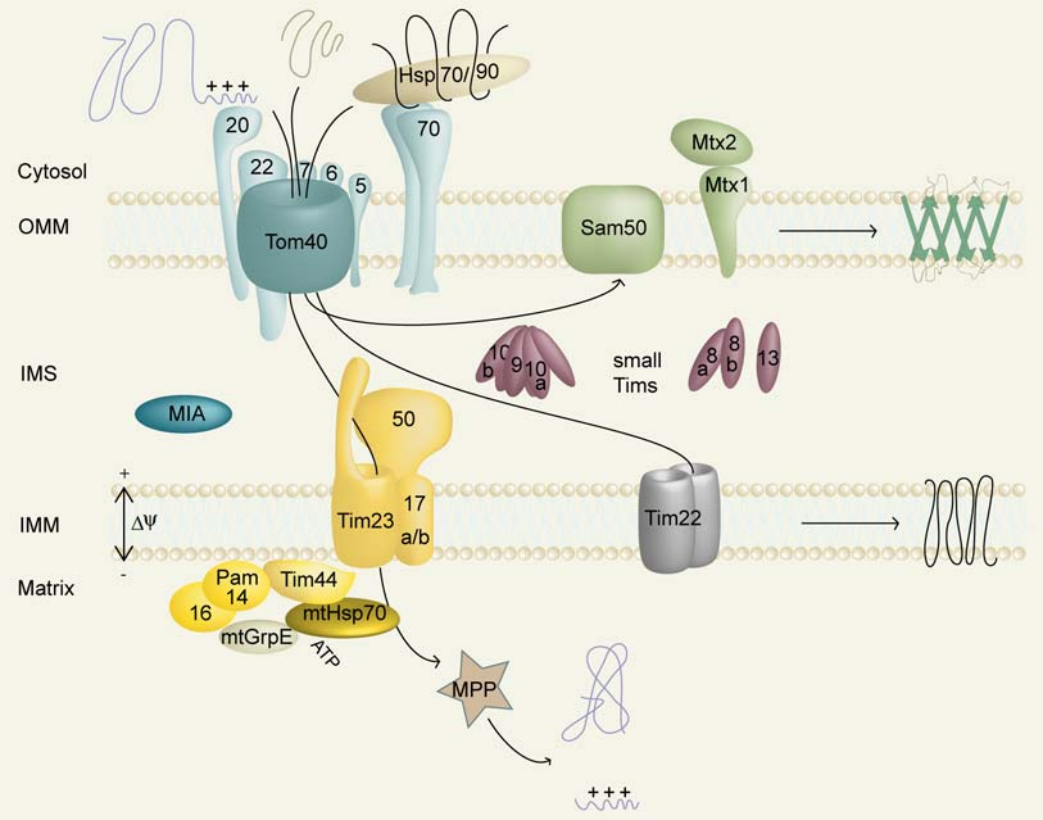

Figure 2-7. Protein import into mammalian mitochondria. The mammalian TOM complex is highly similar to its fungal homologue, with the receptors Tom20, Tom70 and Tom22, the central import pore build by Tom40 and the accessory small Tom proteins Tom5, Tom6 and Tom7. In contrast, the mammalian SAM complex differs from its yeast counterpart. Assembly of β-barrel proteins through the highly conserved Sam50 in mammalian mitochondria is assisted by Metaxin 1 and 2. The mammalian TIM23 translocase resembles the corresponding complex in yeast , although minor differences like the occurrence of two Tim17 genes exist. Import of carrier proteins by TIM22 is not explored in detail in mammals yet, and apart from its central core Tim22, no homologues to the yeast TIM22 complex factors have been found. In the intermembrane space, as shown for yeast, the small Tim proteins Tim8a, 8b, 9, 10a, 10b and 13 build chaperone complexes that guide precursors from the TOM to the SAM or the TIM22 complex. The MIA device in mammals is required for the import of small IMS proteins as in yeast. Hsp, Heat shock protein; IMM, inner mitochondrial membrane; IMS, intermembrane space; MIA, mitochondrial intermembrane space import and assembly; MPP, mitochondrial processing peptidase; Mtx, Metaxin; OMM, outer mitochondrial membrane; PAM, precursor-associated motor; SAM, sorting and assembly machinery; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane; Δψ, mitochondrial inner membrane potential.

even structurally similar, although they seem to be unrelated [Pfanner, 04; Wiedemann, 06]. The Tim proteins Tim23, Tim17, and Tim22 share sequence homology to a family of bacterial amino acid transporters [Rassow, 99]. Family members of Tim44 can be found in all eukaryotes and α-proteobacteria [Walton, 04]. The matrix folding machinery clearly derived from bacterial chaperones, as seen by the high homology of Mge1 and Mdj1 to GrpE and DnaJ, respectively. Further, the

cochaperones Cpn60 and Cpn10 are derived from GroEL and GroES [Ryan, 97], and mtHsp70 originates from bacterial DnaK-type Hsp70 proteins [Boorstein, 94; Moro, 05]. Bacterial homologues were also described for MMP and the inner mitochondrial membrane protease [Braun, 95; Paetzel, 02; Taylor, 01].

The signal sequences targeting the early mitochondrial proteins to the organelle likely stem from bacteria, as hydrophobic, discrete sequences resembling N-terminal presequences can also be found in bacteria [Lucattini, 04]. Moreover, several pathogenic bacterial proteins are able to target mitochondria of host cells [Kozjak-Pavlovic, 08]. Additionally, the cotranslational import of some precursors on mitochondrially bound ribosomes sometimes observed in yeast might represent a relict of early mitochondrial protein targeting [Lister, 06]. It was proposed that the protein import across the OMM in an early protomitochondrial stage occurred through a primitive outer membrane translocation machinery, probably an Omp85 homologue [Dolezal, 06; Lister, 06]. Transport across the IMM might initially have been accomplished via the bacterial sec machinery that was shown to theoretically work in a rethrograde mode [Cavalier-Smith, 06; Romisch, 99]. As soon as the first import devices into mitochondria had developed, gene copies in the mitochondrial genome that were doubled and transferred to the nucleus might have been lost; these events possibly triggered the evolution of mitochondrial import complexes [Cavalier-Smith, 06; Lister, 06]. The TOM complex consisting of Tom40, Tom22, and the small Tom proteins developed, as these factors are present in all eukaryotic mitochondria today [Macasev, 04]. This early TOM complex must have lacked receptors, because Tom20 and Tom70 only are present in fungi and animals, but not in plant mitochondria [Chan, 06; Likic, 05]. Furthermore, import can even occur in the absence of Tom20 and Tom70 in yeast mitochondria, though with slower kinetics [Lithgow, 94]. After separation of yeast cells from the lineage of higher eukaryotes, both types of mitochondria developed additional factors like the metaxins in the mammalian SAM complex or Tim54, Tim18, and Tim12 in the fungal organelle (for comparison of fungal and mammalian import see Table 1 and Figure 2-7).

Table 1: Mitochondrial protein import components (after: Hoogenraad *et al.*, 02)

	Fungi	Mammals
Cytosol	Hsp70, Hsp90	Hsp70/Hsc70, Hsp90/Hsc90
		MSF, PBF
Outer membrane	Tom20	Tomm20/ Tom20
	Tom22	Tomm22/ Tom22
	Tom70	Tomm70/ Tomm70
		Tomm34/ Tom34
	Tom5/ Mom8a	Tomm5/ Tom5
	Tom6/ Mom8b	Tomm6/ Tom6
	Tom7/ Mom7	Tomm7/ Tom7
	Tom40	Tomm40/Tom40
	Sam50/ Tob55	Samm50/Sam50
	Sam35/ Tom38/ Tob38	
	Sam37/ Mas37	Metaxin 1/ Mtx1
	Mdm10	
	Mdm12	
	Mmm1	
	Mim1/ Tom13	
		Metaxin 2/ Mtx2
Intermembrane space	Mia40/ TIM40	Mia40/ CHCHD4
	Erv1	GFER / ALR
	Tim8	Tim8a,b/ DDP1,2
	Tim9	Timm9/ Tim9
	Tim10	Timm10a,b/ Tim10a,b
	Tim13	Timm13/ Tim13
Inner membrane	Tim23/ Mas6/ Mim23	Timm23/ Tim23
	Tim17/ Mim17	Timm17a,b/ Tim17a,b
	Tim21	
	Tim50	
	Tim22	Tim22
	Tim11/ ATP21	
	Tim18	
	Tim54	
	IMP	IMP
Matrix	Tim44/ Mim44	Timm44/ Tim44
	Pam16/ Tim16	Pam16/ Tim16/ Magmas
	Pam17	
	Pam18/ Tim14	Timm14/ Tim14/ DNAJC19
	mtHsp70/ Ssc1	mtHsp70
	Mge1	hTid1
	Mdj1	GrpEL1
	MPP	MMP
	MIP	MIP
	Cpn60/ Hsp60	HSPD1/ Cpn60/ Hsp60
	Cpn10/ Hsp10	HSPE1/ Cpn10/ Hsp10

3 Generation of stable shRNA-knockdown cell lines

3.1 A short introduction to RNA interference

The discovery of RNA interference (RNAi) opened enormous opportunities for both basic research and clinical applications [Lopez-Fraga, 08; Raemdonck, 08]. Since the first description of gene silencing by artificial antisense double-stranded (ds) RNA in *Caenorhabditis elegans* [Fire, 98], functional application of small dsRNA was shown in many organisms including mammalians [Elbashir, 01; Kim, 07]. The different RNAi-based approaches for gene silencing mimic steps of the endogenous microRNA (miRNA) pathway, where genome-encoded miRNA precursors are first processed in the nucleus by Drosha into stem-loop structures and shuttled into the cytoplasm by Exportin-5 [Lund, 04; Yi, 03a; Zeng, 05]. There, pre-miRNAs are processed by the endoribonuclease Dicer into 21 to 23 bp long dsRNA molecules with a two nucleotides overhang at the 3´ ends, a form that is recognized by the RNA-induced silencing complex (RISC) [Kim, 05]. The RISC component Argonaute 2 (AGO2) cleaves the sense RNA strand to generate a single-stranded (ss) antisense RNA that guides RISC to a complementary sequence in the 3´UTR (untranslated region) of the target mRNA and abolishes its translation [Liu, 05]. The RISC complex is then recycled for further specific mRNA silencing [Hutvagner, 02]. RNAi-induced gene silencing is often achieved by transient transfection of synthetic small interfering RNA (siRNA) into the cellular cytoplasm. SiRNA is recognized by RISC, processed by AGO2 and guides the activated RISC complex to the complementary mRNA sequence [Tolia, 07]. In contrast to miRNA-mediated silencing, the endonuclease activity of RISC cleaves the target mRNA, thereby generating unprotected RNA ends that are recognized by intracellular nucleases and degraded [Liu, 04]. Alternatively, short hairpin RNA (shRNA) is expressed that resembles miRNA precursors in the stem-loop structure and the two nucleotides overhang at the 3´ends. Similarly to miRNAs, shRNAs are exported into the cytoplasm by Exportin-5 and processed by Dicer into the form recognized by RISC, leading to target mRNA degradation [Bernstein, 01; Yi, 03a].

For experimental gene silencing, the decision on whether to use siRNA or shRNA greatly depends on the application aimed at. SiRNAs can only be transiently transfected, which limits the duration of gene silencing to few days [Bartlett, 06]. ShRNAs, in contrast, can be expressed continuously from RNA-polymerase driven expression cassettes transferred into cells by viral transduction. These cassettes are either stably integrated into the nuclear genome, if retroviruses or lentiviruses are used, or remain mainly episomal in the case of adenoviral vectors [Cullen, 06; Song, 04; Wiznerowicz, 06]. This form of application also makes gene silencing by shRNA cheaper and easier available compared to siRNA-mediated gene knockdown and, furthermore, minimizes the risk of RNAse degradation. Moreover, viral delivery ensures maximal transduction rates in contrast to the less efficient transfection of siRNA [Wiznerowicz, 06]. In general, shRNA-induced gene silencing achieves a higher knockdown because Dicer, required for the maturation of shRNA - but not of siRNA - was found to participate in early RISC assembly [Lee, 04]. By using siRNA longer than 21 bp that also underlies Dicer processing this obstacle can be circumvented [Kim, 05].

3.2 The lentiviral two-vector system

In the present approach single HeLa cell clones were generated that carry a genome-integrated cassette encoding a shRNA under an inducible polymerase III (Pol III) promoter. The usage of an inducible promoter for shRNA expression is inevitable for the investigation of essential proteins like Tom40 or Sam50. In addition, cells in which the knockdown was not induced provide a perfect control for the parallel investigation of on- and off-status of a certain gene. Moreover, the inducibility of a disadvantageous shRNA can prevent a possible epigenetic silencing in the promoter region by histone modifications and hypomethylation of CpG islands [Mutskov, 04]. Pol III promoters are ideal for shRNA expression as they produce RNA transcripts lacking a polyadenylation tail that would influence processing of the shRNA by Dicer. Termination of shRNA transcription is achieved by adding four or five thymidine residues downstream of the shRNA-encoding sequence [Brummelkamp, 02]. In addition, their small size and their defined transcription start site make Pol III promoters suitable for shRNA approaches [Tuschl, 02; Wiznerowicz, 06]. The Pol III promoter H1 used here for shRNA expression is under the control of an *E. coli* tet operon (*tetO*). Because *tetO* does not guarantee a complete repression of transcription, the tet repressor (tTR) is combined with the Krüppel-associated box (KRAB) domain to achieve epigenetic silencing by heterochromatin formation [Wiznerowicz, 03].

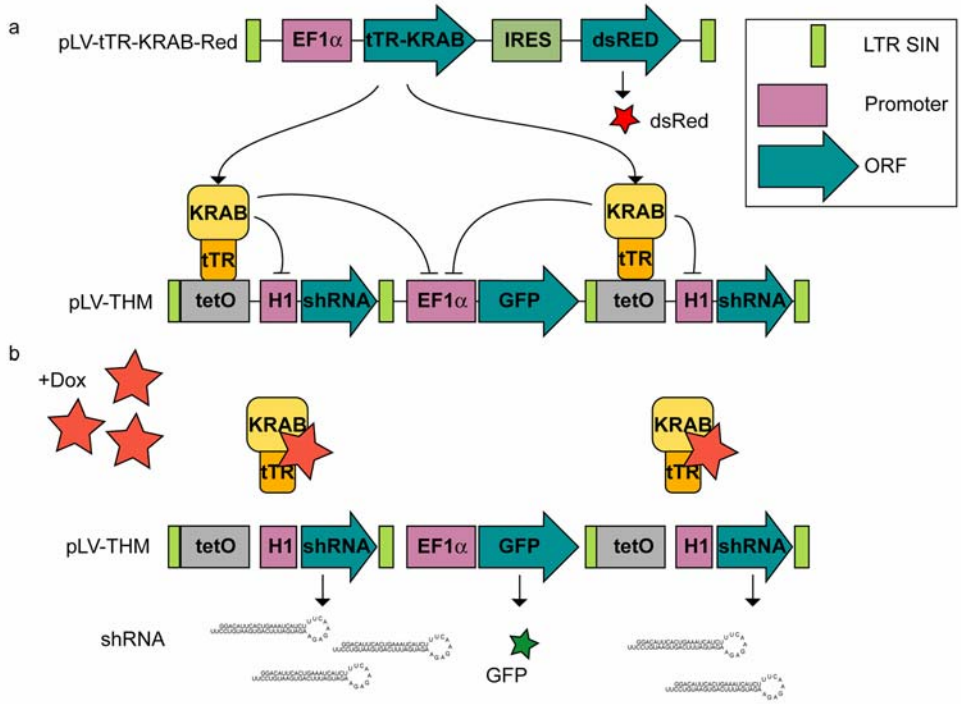

Figure 3-1. The lentiviral two-vector system for conditional shRNA expression. (a) The tTR-KRAB repressor, constitutively expressed from the pLV-tTR-KRAB-Red lentivector as a bicistronic mRNA with dsRed inhibits expression from promoters located 3kb up- or downstream of the tet operon (*tetO*) on the pLV-THM lentivector. (b) Upon addition of doxycycline (+Dox), the tTR-KRAB repressor looses its affinity for *tetO*, and expression of shRNA and GFP from the H1 or EF1α promoter respectively takes place. LTR SIN, self-inactivating long terminal repeat, ORF, open reading frame, IRES, internal ribosome entry site.

Thereby, the resulting fusion protein binds to *tetO* via the tTr element of the repressor, while the Krüppel domain silences promoters located 3kb up- and downstream of the repressor binding site (Figure 3-1a). Addition of doxycycline (Dox) abolishes binding of the repressor to *tetO* and thereby triggers shRNA expression (Figure 3-1b). As the tTR-KRAB repressor can also control polymerase II (Pol II) promoters, the expression of shRNA can be monitored by a GFP marker expressed from the Pol II promoter EF1α placed nearby *tetO*. TTR-KRAB is expressed on a different vector from the constitutively active EF1α promoter as part of a bicistronic transcript also encoding dsRed that is processed posttranscriptionally via an IRES site (Figure 3-1). The lentiviral vectors pLV-THM and pLV-tTR-KRAB-Red are flanked by self-inactivating (SIN) long terminal repeats (LTR) to ensure that lentiviral LTRs are not transcriptionally active. In order to increase shRNA expression, the tetO-H1-shRNA cassette was placed within the 3´ SIN LTR region in pLV-THM and therefore gets dublicated during reverse transcription [Wiznerowicz, 03].

3.3 Results

3.3.1 Validation of shRNA efficiency in cell pools

In order to yield a high knockdown efficiency, shRNA-encoding sequences were designed with 21 nucleotides (nt) forming the shRNA stem interrupted by a 9 nt loop sequence that enables the processing to siRNA with a two bp 3´ overlap by Dicer cleavage (Figure 3-2) [Brummelkamp, 02].

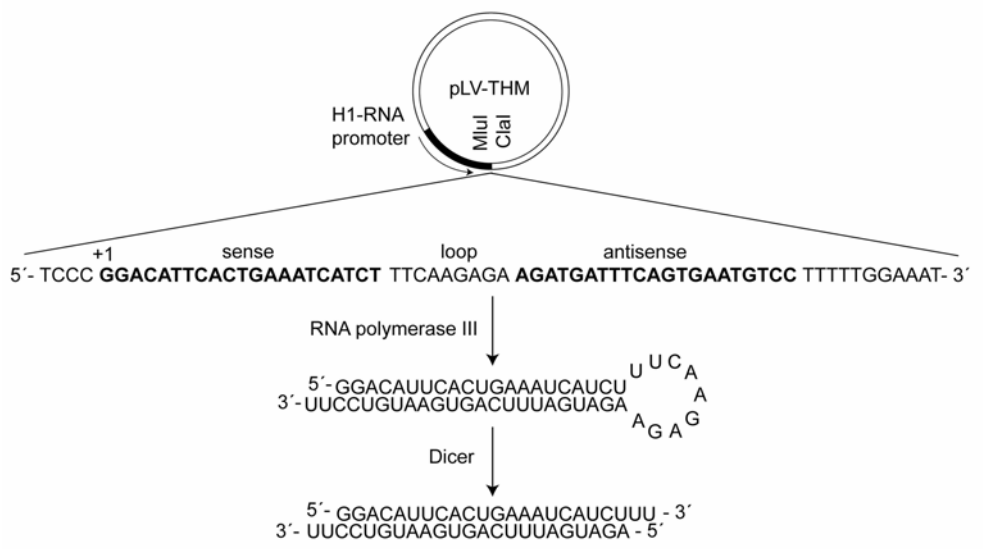

Figure 3-2. Processing of shRNA cloned into the pLV-THM vector. ShRNA-encoding sequences consisting of 21 nucleotides (nt) sense strand, 9 nt loop sequence and 21 nt antisense strand are cloned between MluI and ClaI sites downstream of the H1 promoter. Transcription by RNA polymerase III initiates 9 bp downstream of the promoter (+1) and terminates after the second of five thymidine residues. The resulting shRNA is processed by Dicer to siRNA with two nucleotides overhang at the 3´ ends.

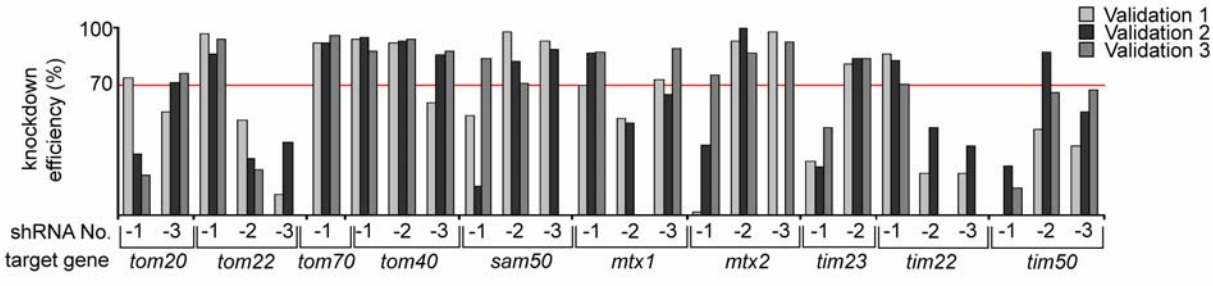

Figure 3-3. Validation of shRNAs in HeLa cell pools by qRT-PCR. In three independent validation experiments, HeLa cells were transduced with lentivirus packed with shRNA-encoding vectors. Cells were lysed, and RNA was isolated. By qRT-PCR using GAPDH as an internal standard, levels of the mRNA targeted by the specific shRNA were assessed. Cells transduced with the empty vector were set as 100 %. ShRNAs are specified by their target gene and the number of shRNA. Mtx1, Metaxin 1; Mtx2, Metaxin 2.

ShRNAs targeting genes of the outer mitochondrial membrane (OMM) import factors Tom20, Tom22, Tom70, Tom40, Sam50, Metaxin 1, and Metaxin 2 or the inner membrane factors (IMM) Tim23, Tim22, and Tim50 were cloned into the pLV-THM vector. Upon sequence verification lentivirus packed with the shRNA-encoding pLV-THM vector was produced and used for the transduction of HeLa cells. As these cells do not encode the repressor, shRNAs are expressed constitutively. In three independent transduction experiments, the knockdown of cell pools was determined by quantitative real-time PCR (qRT-PCR). Validation revealed that the efficiency of knockdown for most shRNAs was very high in HeLa cells (Figure 3-3). ShRNAs with knockdown efficiencies of approximately 70 % or higher in at least two validation rounds were considered as promising candidates for the successful generation of single cell clones.

3.3.2 Selection and validation of single cell clones

For the generation of inducible shRNA cell lines, a cell clone named HeLa-KRAB which stably carries the repressor-encoding vector pLV-tTR-KRAB-Red and which has been generated previously, was utilized. HeLa-KRAB cells were transduced with shRNA-encoding lentivectors or empty pLV-THM vector for control experiments; subsequently, single cell clones were isolated. To this end, only cells that expressed dsRed, but not GFP, were sorted by FACS, as in some cells the promoter seems to be leaky, permitting shRNA and GFP-expression even in the presence of the repressor (Figure 3-4a).

After sorting into 96-well plates, wells containing more than one cell were detected and excluded from further processing to ensure monoclonality. At least 10 cell clones per shRNA were expanded and induced with doxycycline for 7 days. Clones that expressed GFP upon Dox-treatment (Figure 3-4b) were screened for knockdown efficiency by qRT-PCR. The clone with the most efficient RNA knockdown for each shRNA is depicted in Figure 3-4c. The selected clone is named with the target gene, followed by the respective shRNA number. For example a clone with stably integrated shRNA number 2 targeting *tom40* is called *tom40kd-2.* Cell clones with a low RNA level of the gene of

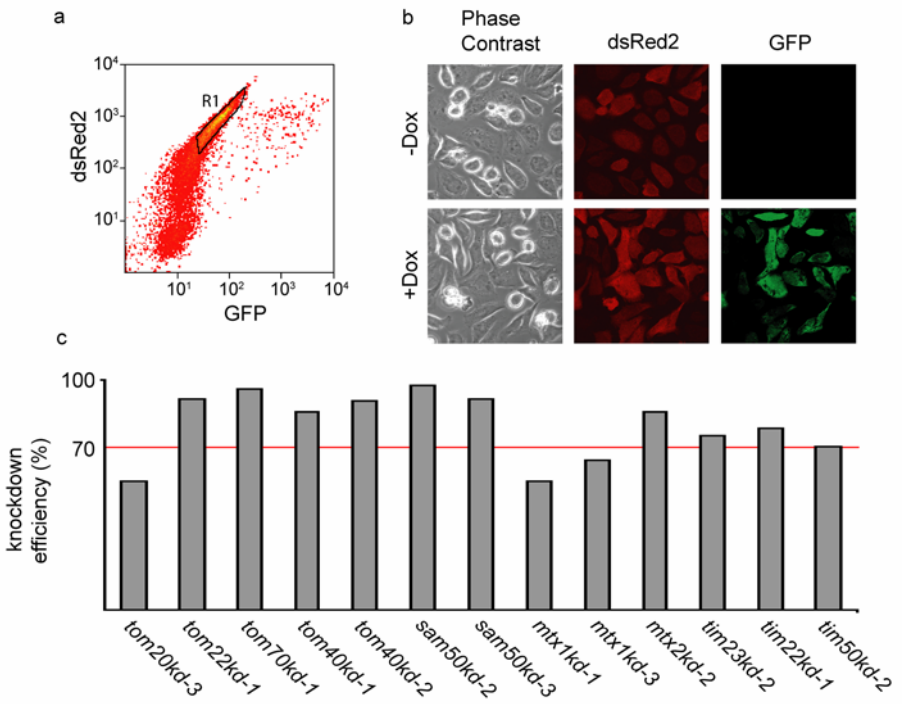

Figure 3-4. Generation of single cell clones. (a) HeLa-KRAB cells transduced with shRNA-encoding pLV-THM lentivectors were sorted by FACS. Single cells expressing dsRed, but not GFP were sorted into 96-well plates for the selection of single cell clones. R1 defines the cell population used for sorting. (b) A single cell clone was induced with 1 µg/ml doxycycline (Dox). After 7 days, expression of GFP and dsRed was monitored by fluorescence microscopy. (c) Single cell clones were induced with doxycycline for 7 days. Then the knockdown of the respective target gene was assessed by qRT-PCR relative to the RNA level in the non-induced cell clone. GAPDH was used as an internal standard. Mtx1, Metaxin 1; Mtx2, Metaxin 2.

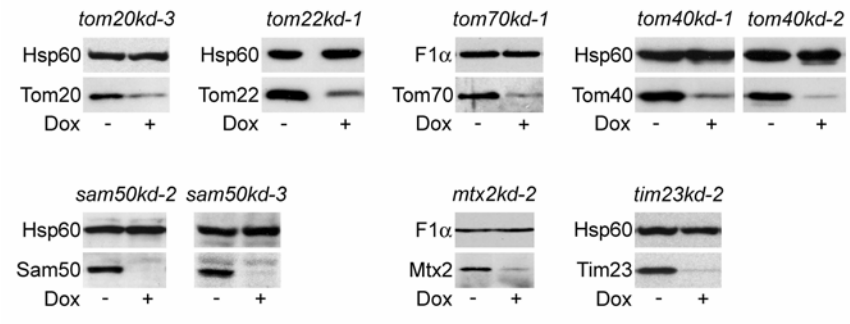

Figure 3-5. Levels of shRNA-targeted proteins in single cell clones. Cell clones that revealed an efficient knockdown on RNA level were induced with 1 µg/ml doxycycline (Dox) for 7 days. Mitochondria were isolated from both non-induced and Dox-induced cells, and protein levels were assessed by applying 50 µg of mitochondrial protein on SDS-PAGE followed by western blot. Antibodies specific for the respective proteins knocked down by Dox-treatment were used, and the levels of mitochondrial Hsp60 or of the α subunit of the ATPase F1 complex (F1α) served as a loading control. Mtx2, Metaxin 2.

interest were then further tested by western blot for the knockdown on protein level (Figure 3-5). Cell lines with a high efficiency in shRNA-mediated protein knockdown upon doxycycline-induction could be gained for all TOM receptor proteins Tom20, Tom22 and Tom70. Both shRNAs targeting the import pore Tom40 achieved a prominent loss in Tom40 protein level. Also, cell clones nearly completely lacking Sam50, the core protein of the β-barrel integrating SAM complex, could be obtained for both shRNAs used. Finally, cell lines with a strong knockdown in Metaxin 2, a protein assisting Sam50, and Tim23, the translocase of matrix proteins, were isolated. Although cell lines with Tim22 and Tim50 shRNAs revealed a strong decrease of the respective RNA upon Dox-induction (Figure 3-4c), they could not be tested on protein level because no antibodies were available for these proteins.

3.3.3 Levels of mitochondrial import factors in knockdown cell lines

The difference between yeast temperature-sensitive mutants generally used for studies on mitochondrial protein import and conditional shRNA-mediated knockdown cell lines is that the induction of RNAi silencing takes several days and potentially affects other proteins whose import depends on the shRNA-targeted factors. If these proteins are themselves participating in protein import, this secondary effect might influence results obtained with these cell lines. Therefore, levels of other mitochondrial proteins were assessed by western blot after inducing the knockdown in the cell lines. Only cell lines that were also used in the following studies are presented here.

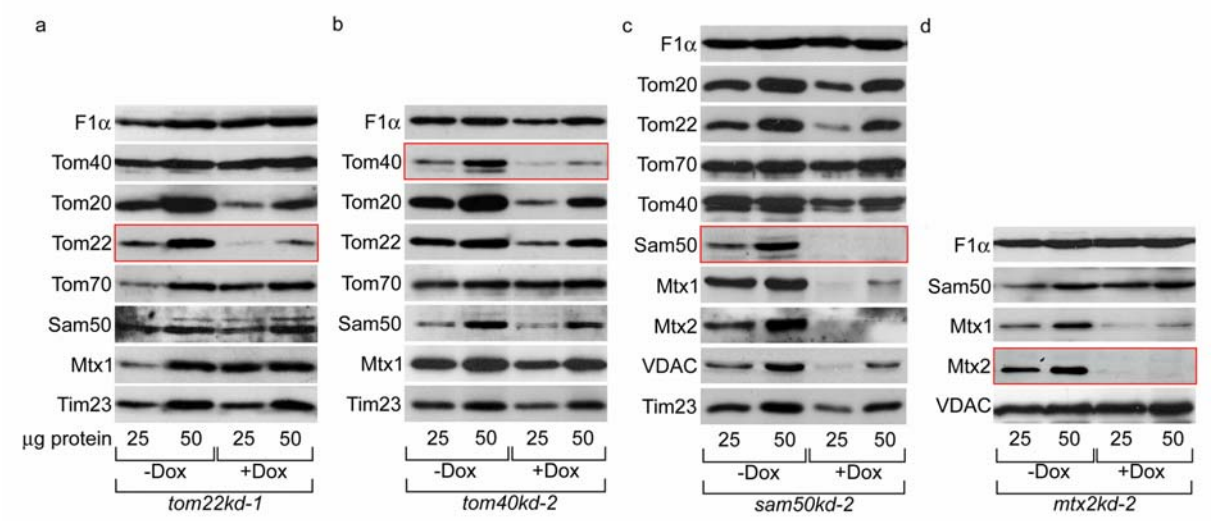

Figure 3-6. Levels of mitochondrial proteins in shRNA knockdown cell lines. (a) *Tom22kd-1*, (c) *sam50kd-2* and (d) *mtx2kd-2* cells were induced with doxycycline (Dox) for 7 days, (b) *tom40kd-2* cells only for 5 days. Mitochondria were isolated from non-induced and induced cells, and 25 or 50 µg of mitochondrial protein as indicated was separated by SDS-PAGE followed by western blot. Mitochondrial protein levels were detected with the respective antibody. Levels of the α subunit of the ATPase F1 complex (F1α) were used as a loading control. The red rectangle marks the protein targeted by the respective shRNA. Mtx1, Metaxin 1; Mtx2, Metaxin 2.

ShRNA-expression in *tom22kd-1*, *sam50kd-2,* and *mtx2kd-2* cells was triggered by cultivating them in the presence of doxycycline for 7 days. *Tom40kd-2* cells were treated with doxycycline for only 5 days, as the knockdown of Tom40 is already very prominent in this cell clone after this period of time, and shorter expression of shRNAs minimizes secondary effects on other proteins. Knockdown of the receptor protein Tom22 had no influence on most of the tested proteins (Figure 3-6a). Only the amount of Tom20 was reduced, consistent with the finding that the Tom22 level is connected to that of Tom20 [Harkness, 94; van Wilpe, 99]. The cell line with an shRNA targeting Tom40 revealed only a very slight reduction in Tom20, Tom22, and Sam50 (Figure 3-6b). In *sam50kd-2* cells the doxycycline-treatment greatly affected the levels of Metaxin 1 and Metaxin 2, proteins assisting Sam50 in the import of β-barrel proteins. In addition, the amounts of β-barrel proteins Tom40 and, to a greater extend, VDAC were reduced upon knockdown-induction. Minor reductions in the levels of Tom22 and Tim23 were observed. (Figure 3-6c). The depletion of Metaxin 2 in the *mtx2kd-2* cell line resulted in a nearly equal reduction of Metaxin 1, while substrate proteins of the SAM complex like Sam50 itself and VDAC remained unchanged (Figure 3-6d).

3.3.4 Integrity of the mitochondrial outer membrane in knockdown cell lines

It was shown before that a destabilization of the OMM affects the import of VDAC [Smith, 94]. To exclude that mitochondria of knockdown cell lines loose their OMM integrity, proteins in the intermembrane space (IMS) were analyzed for protease accessibility. Therefore, the shRNA-mediated knockdown of Tom22, Tom70, Tom40, Sam50, and Metaxin 2 was induced in the appropriate cell line and mitochondria were isolated from these cells. After treatment of mitochondria with proteinase K the levels of the IMS proteins AIF, Tim23, and Smac/DIABLO remained unchanged, while, when the OMM was disrupted by swelling, the tested IMS proteins vanished completely (Figure 3-7). This important result proves that the cell lines generated in this work have an intact OMM even under knockdown conditions and are therefore suitable for mitochondrial protein import experiments.

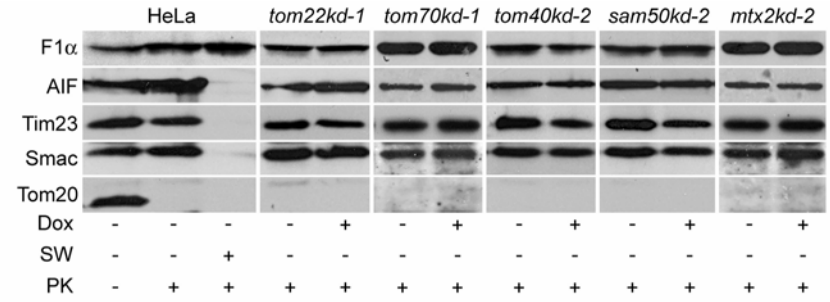

Figure 3-7. Knockdown of import factors does not affect the OMM integrity. In the cell lines *tom22kd-1*, *tom70kd-1*, *tom40kd-2, sam50kd-2* and *mtx2kd-2* knockdown was induced by doxycycline (Dox)-treatment. Mitochondria from non-induced and induced cells were isolated and treated with proteinase K (PK). As control, HeLa mitochondria were undertaken swelling (SW) before PK-treatment. Upon separation by SDS-PAGE and western blot, levels of the IMS proteins AIF, Tim23 and Smac were assessed to control membrane integrity. Tom20 served as control for PK-activity, and F1α as a loading control. Smac, Smac/DIABLO.

3.4 Discussion

RNAi is a powerful alternative to murine knockout systems for most experimental approaches in mammalian systems. It is easier to obtain, and loss of essential proteins can be achieved by using conditional knockdown systems. The cell lines with an inducible shRNA-mediated knockdown of mitochondrial protein import factors generated for this work provide a perfect tool for the investigation of mitochondrial import in mammalian cells. Although initially time consuming, the usage of shRNA cell lines instead of transient siRNA transfection provides the advantage of a continuous system with constant knockdown efficiency. It is also a well-priced alterative to siRNA-mediated knockdown, as protein import experiments often require the isolation of mitochondria from a large number of cells. The inducibility of the lentivectors enables the usage of non-induced cells of the same cell clone as a proper control with an identical genetic background.

There are, however, several points that must be considered when establishing such a system. RNAi was reported to affect the expression of other genes than the targeted one, either by sequence similarity to other mRNAs or by interfering with miRNA-regulated untranslated regions. Therefore, care must be taken if experiments are carried out that monitor a complex phenotype induced by RNAi knockdown - like for example the influence of a certain protein on apoptosis - and more than one shRNA or siRNA should be used for such experiments. Off-target effects can also occur by a saturation of components of the miRNA maturation pathway like Exportin-5 or Dicer [Grimm, 06]. In order to avoid this saturation effect, shRNAs should be used in a way that minimal expression level yields in a maximal knockdown. Here, the inducibility of the used system permits a tight control of shRNA expression. Moreover, a low virus titer was used for transduction of HeLa-KRAB cells to statistically obtain only one lentiviral integration event per cell. The stable genomic integration of lentivectors into the target cell nucleus brings about the potential deletion of important genes. Again, a low virus titer minimizes this risk, and the usage of an inducible promoter for shRNA expression provides a control for off-target effects derived from gene disruption by virus integration. DsRNA used for RNAi can also provoke activation of an interferon type I response by triggering antiviral detectors like PKR or members of the TLR family, leading to the downregulation of gene expression and the onset of apoptosis. On that account, shRNA stem length must be smaller than 30 nt [Lopez-Fraga, 08; Raemdonck, 08]. Although high levels of small dsRNAs induce antiviral innate immune responses, the 21 nt stem length expressed in moderate amounts here minimizes the risk of inducing an interferon response.

In summary, the approach to generate a mammalian system for the study of protein import into mitochondria successfully yielded nine single cell clones with an inducible, shRNA-mediated reduction of proteins implicated in mitochondrial protein import. Additionally, two cell clones with a high reduction in RNA level of the import factors Tim22 and Tim50 were isolated whose protein knockdown needs to be confirmed as soon as appropriate antibodies are available. Five of these cell lines were used in the subsequent projects carried out within the frame of this work and therefore were characterized further with regard to the levels of several mitochondrial proteins and OMM integrity (Figure 3-7). Levels of mitochondrial proteins in *tom70kd-1* cells were not addressed in the present study, as they were shown elsewhere to be unaltered [Kozjak-Pavlovic, 07].

4 TOM-independent complex formation of Bax and Bak

4.1 A short introduction to apoptosis

In most cell types, the mitochondrial outer membrane permeabilization (MOMP) is the irreversible event that leads to the execution of apoptosis [Chipuk, 08b]. Despite extensive research in this field, the molecular nature of MOMP is still unknown. What became clear, however, is that the oligomerization of two major players in the mitochondrial pathway of apoptosis, the Bcl-2 proteins Bax and Bak, in the outer mitochondrial membrane (OMM) is the key event leading to MOMP [Wei, 01; Zong, 01]. The triggers that initiate MOMP are divided into two groups: Extrinsic stimuli that require the extracellular binding of a ligand to the respective receptor and intrinsic initiator events like DNA damage or growth factor withdrawal (Figure 4-1).

The consequence of MOMP is the release of proapoptotic proteins like cytochrome *c* that are sealed in the mitochondrial intermembrane space (IMS) [Scorrano, 02; Yang, 97]. Once liberated into the cytoplasm, cytochrome *c* gains access to Apaf-1 and initiates an ATP-dependent conformational change followed by the oligomerization of Apaf-1 to form the apoptosome. This structure binds to procaspase-9, permits its dimerization and autoproteolytic cleavage. Activated caspase-9 renders the active effector caspases 3 and 7, which in turn promote the degradation of various cellular targets and the packaging of the apoptotic cell for phagocytic removal [Li, 97; Srinivasula, 98, Zou, 97]. Negative regulators of caspase-3 activation, the IAP proteins that interlock several caspases, are inactivated by Smac/DIABLO that gets released from the IMS upon MOMP [Deveraux, 98; Du, 00].

4.1.1 Extrinsic and intrinsic apoptosis pathways

The extrinsic apoptosis pathway is activated by various ligands (e.g. TNFα, TRAIL, or Fas) and results in the formation of DISC, the death inducing signaling complex: upon ligand-binding, the corresponding receptor of the TNFR (TNF receptor) superfamily (e.g. TNFR-1, TRAIL receptor, or CD96 /FAS), homooligomerizes and recruits the adapter molecule FADD (Fas-associated death domain) via an interaction between the death domains of both proteins [Boldin, 95; Kischkel, 95; Wajant, 98]. TNFR-1 signaling requires the additional adaptor protein TRADD (TNFR associated death domain), from which alternative prosurvival pathways branch off [Hsu, 95; Hsu, 96]. DISC assembly then occurs for all receptor types by FADD-mediated binding of procaspase-8. By autoproteolytical cleavage caspase-8 becomes activated and gets released into the cytoplasm [Medema, 97]. The effect of active caspase-8 depends on its concentration. So-called “type I cells” contain high levels of active caspase-8; in this cell type, the effector caspases 3 and 7 are directly triggered by caspase-8. Alternatively, in “type II cells” with a low concentration of active caspase-8, activation of effector caspases involves the mitochondrial pathway [Scaffidi, 98; Scaffidi, 99]. The cleavage of Bid by active caspase-8 results in tBid, which in turn triggers the oligomerization of Bax and Bak [Eskes, 00; Li, 98; Luo, 98; Wei, 00].

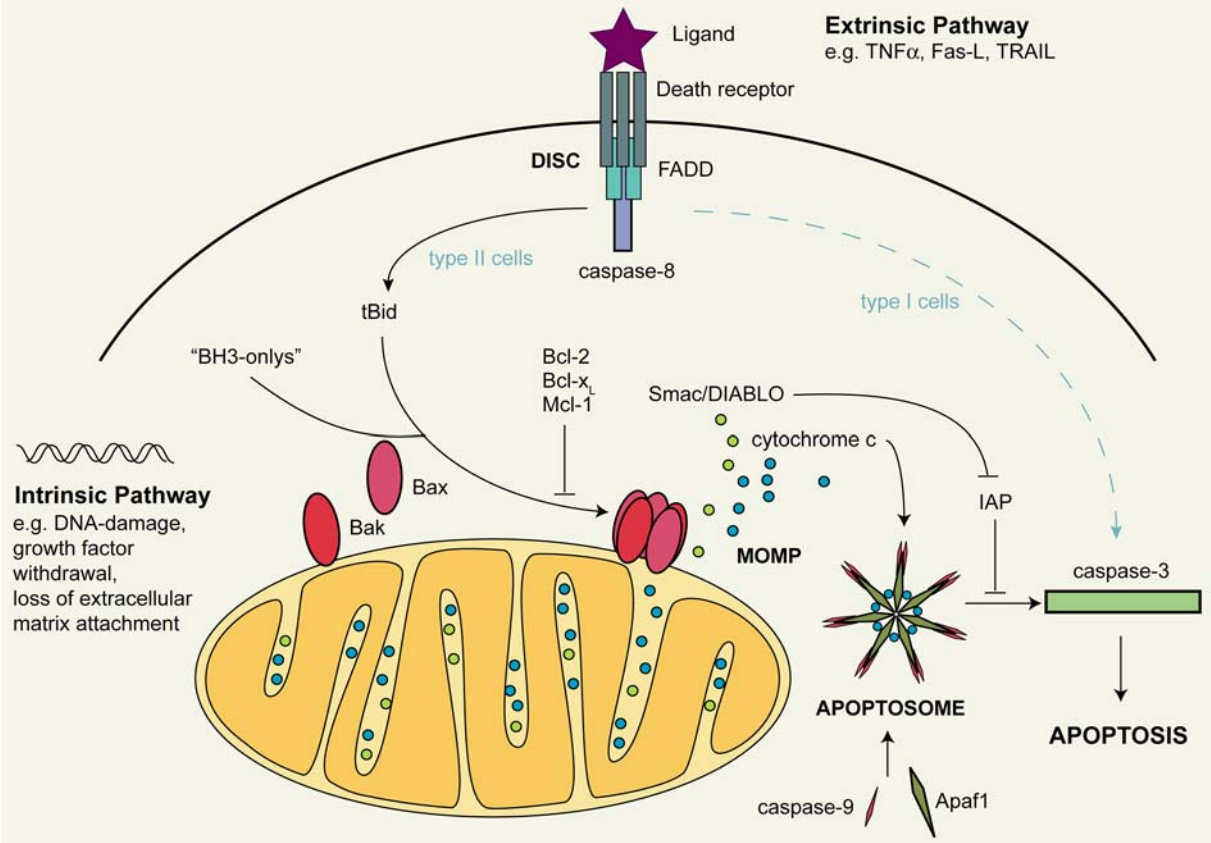

Figure 4-1. Pathways induced by extrinsic or intrinsic apoptosis stimuli. Extrinsic stimulation of apoptosis by death ligands like TNFα or Fas ligand trigger the formation of the death-induced signaling complex (DISC) at the respective receptor. DISC formation results in activation of caspase-8. In type I cells, caspase-8 directly cleaves effector caspase-3 that degrades various cellular targets. In type II cells, due to low concentrations of active caspase-8, amplification of apoptotic signals via mitochondria is required. Caspase-8 cleaves Bid into its truncated form (tBid), resulting in the activation and oligomerization of Bax and Bak in the outer mitochondrial membrane and to subsequent mitochondrial outer membrane permeabilization (MOMP). Intrinsic pathways of apoptosis are initiated by stress signals like DNA damage or growth factor withdrawal. Stress sensors, the BH3-only proteins ("BH3-onlys") respond to these signal by triggering the oligomerization of Bax and Bak and thereby MOMP. This can be inhibited by antiapoptotic Bcl-2 family proteins like Bcl-2 or Bcl-x_L. MOMP results in the release of proapoptotic factors from the mitochondrial intermembrane space, amongst others cytochrome *c* and Smac/DIABLO. Cytosolic cytochrome *c* allows the formation of the apoptosome by Apaf-1, resulting in caspase-9 activation, subsequent effector caspase activation and cellular degradation. Another factor released during MOMP, Smac/DIABLO, deactivates IAPs that block caspase function.

Intrinsic pathways that trigger apoptosis originate from various stress stimuli including DNA damage, growth factor withdrawal or loss of attachment to the extracellular matrix [Fernandez-Luna, 08; Keeble, 07; Youle, 08]. These diverse signals are forwarded by various pathways, involving e.g. p53, MAPK/JNK, and PI3K/AKT, to three groups of Bcl-2 proteins that control MOMP. The proapoptotic effector proteins Bax, Bak, and in few cell types Bok, containing the Bcl-2 homology (BH) domains BH1 to BH3, directly lead to MOMP by oligomerizing in the OMM. This can be inhibited by the

antiapoptotic Bcl-2 proteins, e.g. Bcl-2, Bcl-x_L, and Mcl-1 that have the BH1 to BH4 domains in common. The proapoptotic BH3-only proteins, a third group of Bcl-2 family members that only share the BH3 domain, are often referred to as the sentinels of programmed cell death, because each of them senses certain types of cellular stress. DNA damage for example leads to the upregulated expression of the BH3 only proteins Puma and Noxa via p53 [Nakano, 01; Oda, 00], while growth factor withdrawal can activate Bad and Bim that are normally suppressed by phosphorylation via Akt or ERK [del Peso, 97; Ley, 03]. The mode of apoptosis control between these three groups is still a matter of debate. It was suggested that the executioner proteins Bax and Bak are permanently kept in check by antiapoptotic antagonists in healthy cells. Upon activation, the BH3-only proteins would neutralize antiapoptotic proteins and thereby free Bax and Bak, leading to MOMP [Fletcher, 08; Willis, 05; Willis, 07]. Another hypothesis considers that Bid and Bim directly activate the effectors Bax and Bak and therefore are called “activators” in contrast to the other BH3-only proteins, termed “derepressors”. According to this model antiapoptotic factors sequester the activators Bid and Bim in healthy cells. Upon initiation of intrinsic cell death, derepressors liberate activators and permit the activation of effectors [Cheng, 01; Chipuk, 08a; Kuwana, 05]. However, several evidences indicate a participation of additional non-Bcl-2 proteins in MOMP regulation [Chipuk, 08b; Keeble, 07].

4.1.2 Targeting of mitochondria by Bax and Bak

Although the requirement of Bax and Bak oligomerization for MOMP is well documented, details about the targeting of mitochondria by Bax and Bak are hardly known. Bax and Bak are tail-anchored mitochondrial proteins [Cheng, 03; Goping, 98; Nechushtan, 99; Wolter, 97]. This group of outer membrane proteins contains a carboxy (C)-terminal targeting signal that forms the transmembrane segment (TMS). Lacking any sequence conservation, common structural features among tail-anchored proteins are moderate hydrophobicity of the TMS with positive charges at its flanking regions [Borgese, 07; Rapaport, 03]. Under non-apoptotic conditions Bax is a soluble cytosolic protein, with its C-terminal hydrophobic TMS residues hidden within a hydrophobic pocket in the protein. Upon induction of apoptosis, the TMS becomes exposed and results in translocation of Bax into the OMM [Desagher, 99; Nechushtan, 99; Wolter, 97]. Bak, in contrast, is permanently localized in the OMM, probably sequestered by Mc-1 and Bcl-xL in an inactive conformation in non-apoptotic cells [Cuconati, 03; Willis, 05]. Activation of both Bax and Bak entails structural rearrangements that lead to the exposition of their amino (N)-terminal domains and oligomerization in the OMM [Antonsson, 01; Brooks, 07; Desagher, 99; Griffiths, 99]. The nature of these Bax and Bak complexes is unknown and was both described as large homo- or heterooligomers between Bax and Bak in large clusters at the OMM [Nechushtan, 01; Sundararajan, 01; Zou, 97]. Oligomers might form a pore that mediates MOMP, although this possibility remains controversial [Kinnally, 07].

As most mitochondrial proteins are considered to require the mitochondrial import machinery to traverse the OMM, its participation in apoptosis was suggested several times [Milenkovic, 04; Motz, 02; Paschen, 03; Pfanner, 04]. The TOM receptor protein Tom22 and the central pore Tom40 were both shown to mediate Bax import into mitochondria [Bellot, 07; Cartron, 08; Ott, 07]. However, another report challenged this view [Sanjuan Szklarz, 07]. Bak integration into the OMM was reported

to require VDAC2 [Setoguchi, 06]. The potential problem with these reports is that most of the authors used yeast as a model system, which particularly in view of the lack of Bcl-2 proteins in yeast mitochondria raises doubts if apoptosis in yeast is sufficiently similar to mammalian cell death [Cheng, 08]. Moreover, frequently the release of cytochrome *c* was monitored rather then Bax or Bak integration into mitochondria. Even when the Bax and Bak translocation to mitochondria was addressed, little care was taken to discriminate between proteins associated with or integrated into the OMM. Finally, the dependency of membrane integration of tail-anchored OMM proteins on the mitochondrial import machinery was questioned recently [Kemper, 08].

In order to shed light on the discussion about a possible participation of mitochondrial import factors in apoptosis, the shRNA cell lines with inducible knockdowns in components of the TOM or the SAM complex produced in this work were applied to study Bax and Bak import. Two approaches were used: the formation of Bax and Bak oligomers upon apoptosis induction was monitored in the absence of mitochondrial import factors of the TOM and the SAM complex. In addition, *in vitro* import experiments were performed under knockdown conditions using the aforementioned cell lines.

4.2 Results

4.2.1 Levels of pro- and antiapoptotic proteins are not affected in shRNA knockdown cells

The ability of Bax and Bak to form complexes in the OMM was found in some reports to depend on the levels of antiapoptotic Bcl-2 proteins [Cuconati, 03; Mikhailov, 01; Willis, 05; Yi, 03b]. Therefore, levels of several proteins involved in apoptosis were monitored in the shRNA knockdown cell lines to control their suitability for the subsequent apoptosis experiments. In the cell lines *tom22kd-1*, *tom70kd-1*, *tom40kd-2*, *sam50kd-2*, and *mtx2kd-2*, shRNA expression was induced with doxycycline, and the levels of antiapoptotic Bcl-2 proteins Bcl-xL and Bcl-2 and of the proapoptotic factors AIF, Smac/DIABLO and cytochrome *c* were compared between mitochondria isolated from non-induced and from induced cells. Despite very efficient knockdowns of the shRNA-targeted proteins in each cell line, no significant changes in the levels of anti- and proapoptotic proteins were detected. Only a slight decrease of cytochrome *c* upon Tom40 knockdown was observed (Figure 4-2). In conclusion, these cell lines are suitable for apoptosis experiments.

4.2.2 Oligomerization of Bax and Bak does not depend on the TOM and SAM complex

In order to address the question of whether the formation of high molecular weight complexes of Bax and Bak in the OMM depends on components of the mitochondrial import machinery, first, the detection of these complexes by blue native-PAGE (BN-PAGE) was established. To this end, apoptosis was initiated in HeLa cells by TNFα and cycloheximide. Like most cells, HeLa are type I cells and respond to TNFα by tBid-mediated oligomerization of Bax and Bak. Mitochondria from apoptotic and healthy cells were solubilized with the mild detergent digitonin to preserve the native membrane

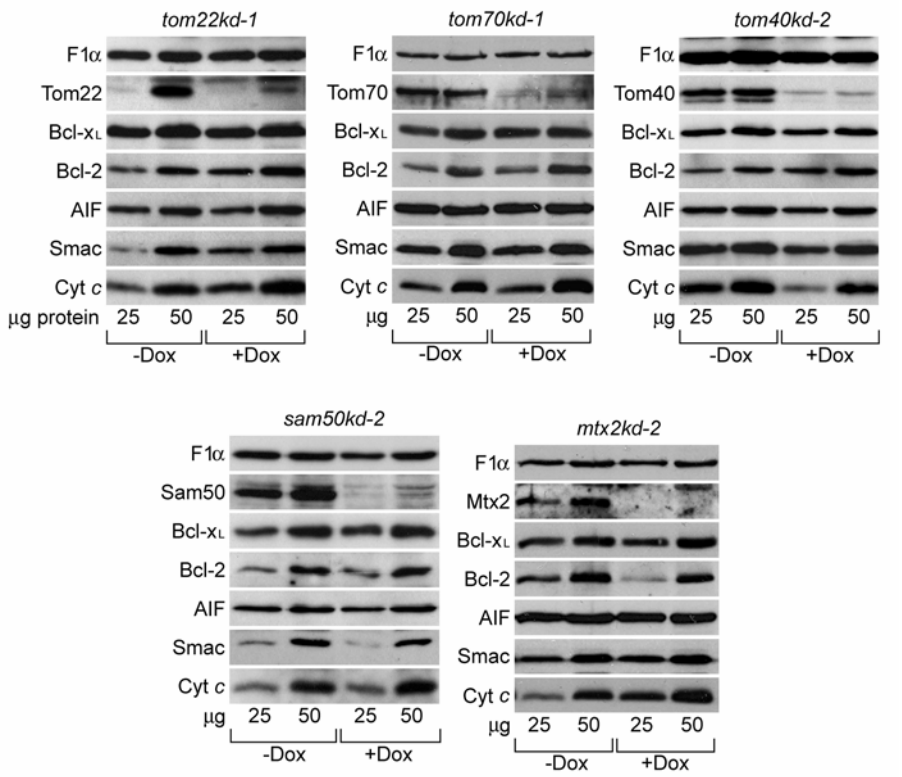

Figure 4-2. Levels of pro- and antiapoptotic proteins in shRNA knockdown cell lines. In *tom22kd-1*, *tom70kd-1*, *sam50kd-2* and *mtx2kd-2* cells, knockdown was induced by growing cells in the presence of 1 µg/ml doxycycline (Dox) for 7 days, *tom40kd-2* cells were induced for 5 days. Mitochondria were isolated from both non-induced (-Dox) and induced (+Dox) cells, and 25 or 50 µg of mitochondrial protein as indicated was separated by SDS-PAGE, followed by western blot. Levels of the antiapoptotic proteins Bcl-x_L and Bcl-2, proapoptotic factors AIF, Smac/DIABLO and cytochrome *c* and shRNA-targeted proteins as indicated in the figure were detected by the appropriate antibody. Amounts of F1α served as a loading control. Mtx2, Metaxin2; cyt *c*, cytochrome *c*.

complexes and were separated by BN-PAGE. By immunoblot, several protein complexes were detected. While in the non-apoptotic sample, the Bax antibody did not detect any protein complex, mitochondria from apoptotic cells revealed various high molecular weight complexes in the range of 66 to 669 kDa (Figure 4-3a). In contrast, Bak was found as a complex pair comigrating approximately with the 440 kDa marker in mitochondria of healthy cells. The amount of this duplex was considerably reduced in the apoptotic sample, while a smaller complex pair in the range of 100 kDa accumulated. The detected complexes were completely membrane-integrated, as both Bax detected in the apoptotic mitochondria and Bak in healthy and apoptotic mitochondria turned out to be resistant to carbonate extraction, like the integral membrane protein Tom40. The soluble matrix protein Hsp60, in contrast, was extracted by the alkaline treatment (Figure 4-3b).

In the following, BN-PAGE was applied to address whether the formation of Bax and Bak complexes would depend on the presence of components of the mitochondrial TOM or SAM complexes. Apoptosis was induced in the cell lines *tom22kd-1*, *tom70kd-1*, *tom40kd-2*, *sam50kd-2,* and *mtx2kd-2* by TNFα and cycloheximide in both control and doxycycline-treated cells, and native complexes of Bax

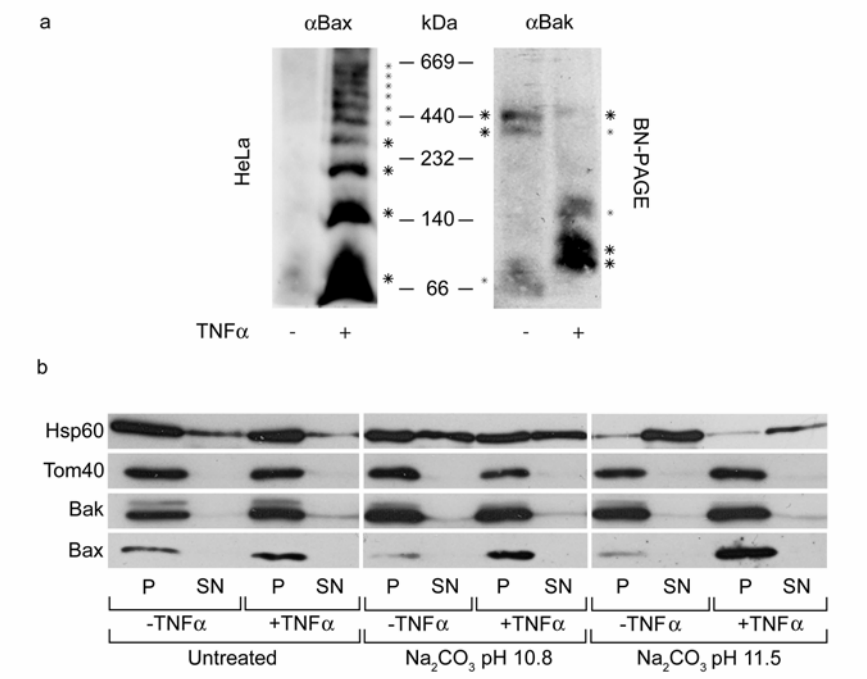

Figure 4-3. Bax and Bak form high molecular weight complexes integrated into the outer mitochondrial membrane. In HeLa cells, apoptosis was induced by 25 ng/ml TNFα and 10 µg/ml cycloheximide. (a) Mitochondria isolated from healthy (-TNFα) and apoptotic (+TNFα) cells were separated by BN-PAGE, followed by western blot. Bax and Bak were detected using the respective antibodies. The more prominent complexes are marked with large, the less prominent complexes with small asterisks. (b) Control and apoptotic mitochondria were treated with 100 mM sodium carbonate at pH 10.8 or 11.5 as indicated. After ultracentrifugation, the detergent-resistant pellet fractions (P) and the TCA-precipitated supernatants (SN) were applied to SDS-PAGE and western blot, and protein levels were assessed with a corresponding antibody.

and Bak were monitored by BN-PAGE. To ensure equal loading on the native gels, F1α levels of each sample were assessed by applying the same amount of mitochondrial protein on an SDS-gel. Despite high knockdown efficiencies in doxycycline-treated samples, the formation of the Bax complexes upon TNFα-treatment was not affected by a knockdown of TOM or SAM complex components (Figure 4-4). Similarly, the Bak complexes in control or apoptotic samples remained unaltered by doxycycline-treatment in the used cell lines. Only in *sam50kd-2* cells, a very slight defect in the assembly of Bax and Bak complexes upon TNFα-treatment was observed (Figure 4-4d).

The induction of shRNA-mediated knockdown for 7 days resulted in a strong reduction of the target proteins (Figure 4-4). However, potential slow turnover rates of Bak could mask a possible effect of protein knockdown on Bak translocation into mitochondria. In order to maximize knockdown efficiencies and to increase secondary effects entailed by a lack of import proteins on other mitochondrial factors, *tom22kd-1*, *tom70kd-1*, *mtx2kd-2*, and *sam50kd-2* cells were treated with doxycycline for 28 days. For *tom40kd-2* cells this long-term treatment with doxycycline was not possible, as these cells stopped growing soon after induction. The long-term depletion especially increased the knockdown of Tom22 in the respective cell line (Figure 4-5a). As before, however, no differences in

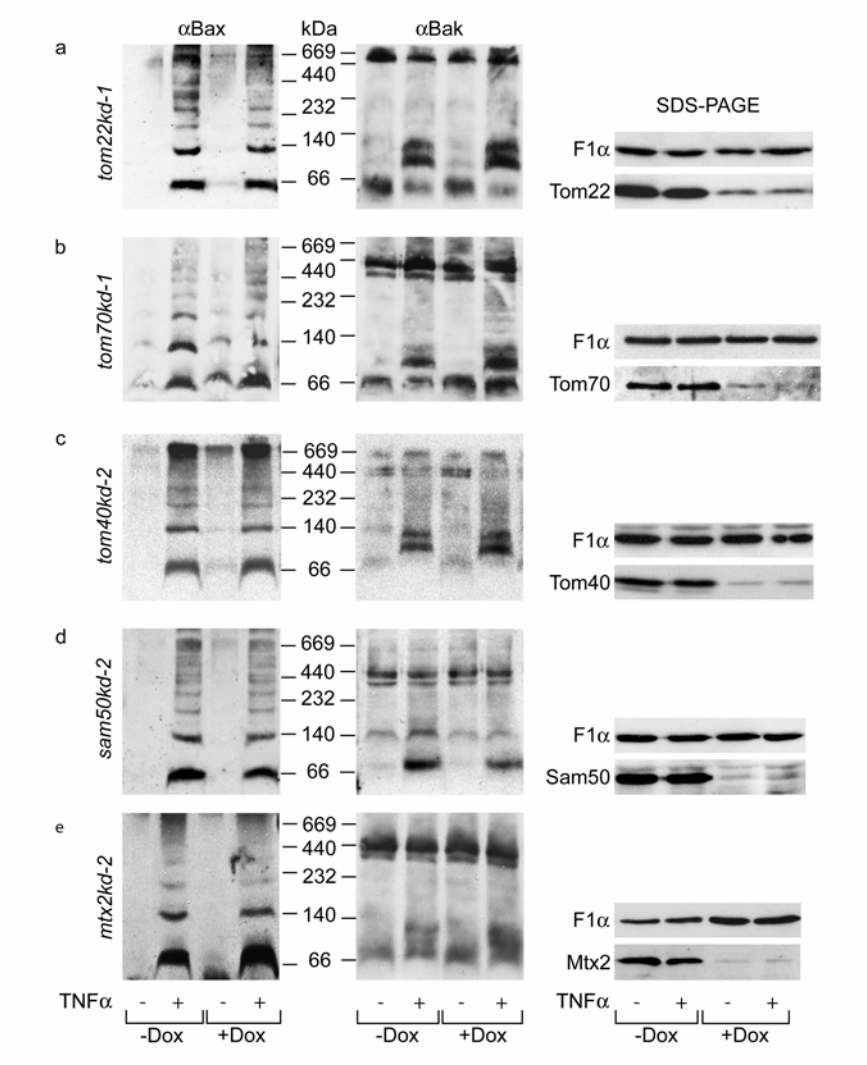

Figure 4-4. Knockdown of TOM or SAM complex proteins does not impair the formation of Bax or Bak complexes. After treatment of the cell lines (a) *tom22kd-1*, (b) *tom70kd-1*, (c) *tom40kd-2*, (d) *sam50kd-2*, and (e) *mtx2kd-2* with doxycycline (Dox) for 7 days, apoptosis was induced with TNFα and cycloheximide in untreated (-Dox) and Dox-treated (+Dox) cells. Mitochondria were isolated from apoptotic (+TNFα) and control (-TNFα) cells and separated by BN-PAGE followed by western blot. Complexes were determined using specific Bax and Bak antibodies. The same mitochondria used for BN-PAGE were applied on SDS-PAGE, where levels of F1α and of the knocked down protein were assessed by immunoblot.

Bax and Bak complex levels under knockdown conditions in TNFα-treated or untreated samples could be detected (Figure 4-5a-c). Only in the case of Sam50, a decrease of Bax complex assembly occurred, being more clearly visible upon extended depletion of Sam50 than after 7 days knockdown. A similar effect was seen for Bak after TNFα-treatment, but not in the healthy cells (Figure 4-5d). By quantification of the four lowest, most prominent Bax complexes on three different native gels, this effect was found to be reproducible (Figure 4-5e). A previously published effector on Bak import and substrate

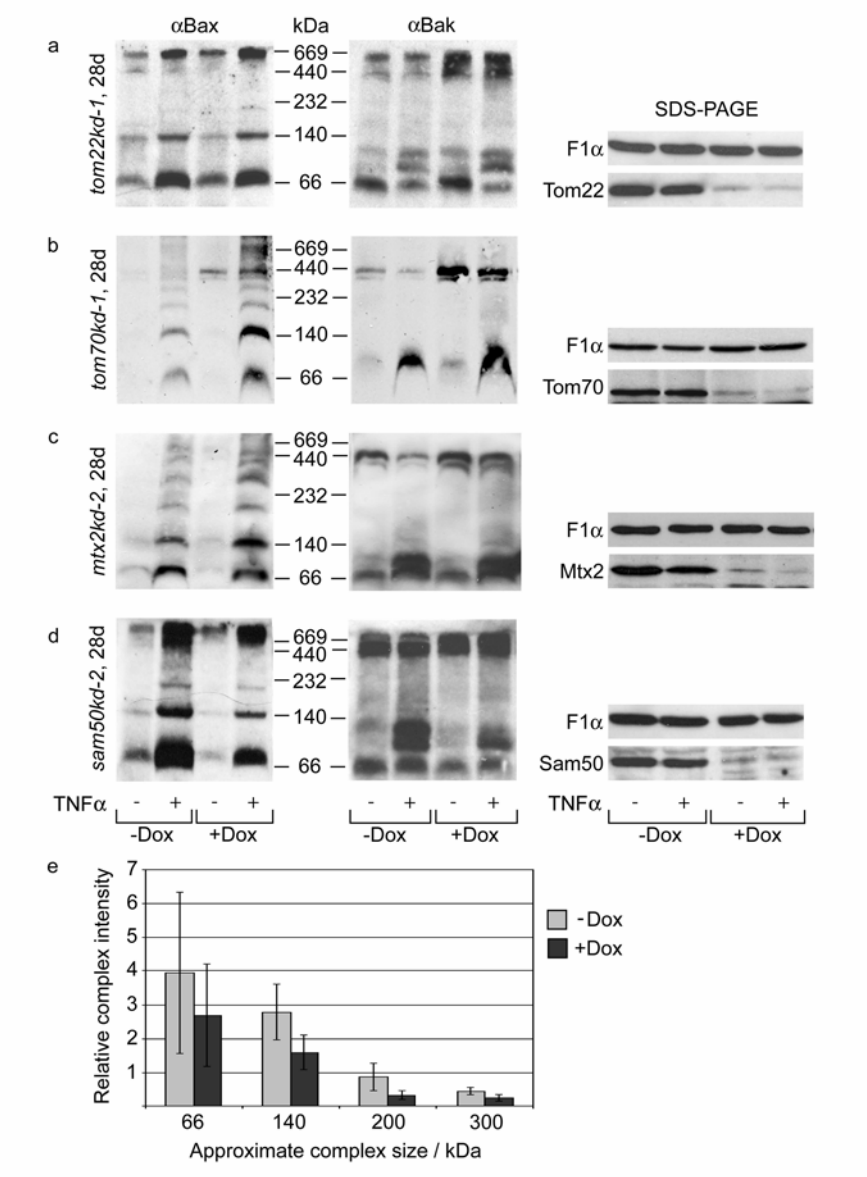

Figure 4-5. Long-term knockdown of Sam50, but not of Tom22, Tom70 or Mtx2 decreased levels of Bax complexes. Cell lines (a) *tom22kd-1*, (b) *tom70kd-1*, (c) *mtx2kd-2* and (d) *sam50kd-2* were treated with doxycycline (Dox) for 28 days (+Dox) or left untreated (-Dox). Upon induction of apoptosis with TNFα and cycloheximide, isolated mitochondria from control (-TNFα) or apoptotic (+TNFα) cells were subjected to BN-PAGE or SDS-PAGE and western blot. (e) Levels of 4 Bax complexes with an approximate size of 66, 140, 200 and 300 kDa were quantified from three independent experiments using the Image J software.

of the SAM complex is VDAC2 [Kozjak-Pavlovic, 07; Setoguchi, 06]. However, levels of VDAC proteins detected by an antibody that recognizes all three isoforms of VDAC, though diminished in *sam50kd-2* cells after 7 and 28 days of doxycycline-treatment, did not differ that much between the two knockdown conditions compared (Figure 4-6), speaking against an influence of VDAC levels on the formation of Bax and Bak complexes.

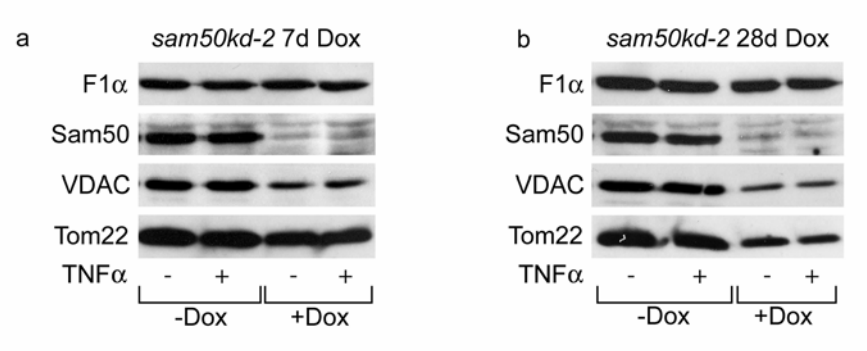

Figure 4-6. Mitochondrial protein levels in *sam50kd-2* cells after Sam50 depletion for 7 or 28 days. The cell line *sam50kd-2* was treated with doxycycline (Dox) for (a) 7 or (b) 28 days. Mitochondria from untreated (-Dox) or treated (+Dox) cells were isolated and separated by SDS-PAGE. Levels of the indicated mitochondrial proteins were detected by the appropriate antibody after western blot.

4.2.3 *In vitro* import of Bax and Bak is not affected by TOM or SAM complex components

In vitro import of radioactively labeled proteins synthesized in rabbit reticulocyte lysate is the method of choice when investigating protein import into mitochondria, as only proteins that *de novo* translocate into mitochondria are detected. Therefor, Bax and Bak import into isolated mitochondria was established. *In vitro* import of VDAC1 served as a control due to its well characterized import pathway [Humphries, 05; Kozjak-Pavlovic, 07]. Because Bax, in contrast to Bak, only translocates to mitochondria upon its activation during apoptosis, a previously described constitutively active variant of Bax with a single point mutation in the C-terminus was used, termed Bax S184V [Nechushtan, 99].

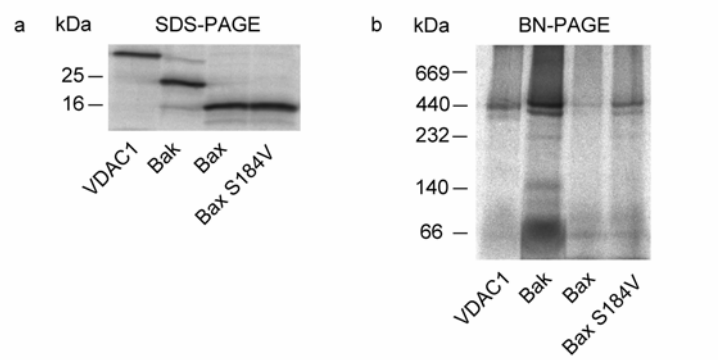

Figure 4-7. *In vitro* transcription and import of Bax, Bax S184V, Bak and VDAC1 into mitochondria. (a) VDAC1, Bak, Bax and Bax S184V genes were expressed *in vitro* in rabbit reticulocyte lysate in the presence of ^{35}S-labeled methionine. 2 µl of each lysate were separated on SDS-PAGE and visualized by radiography. (b) Lysates were imported into 50 µg of isolated HeLa mitochondria for 30 minutes at 37 °C or 25 °C in the case of VDAC1. Mitochondria were then separated by BN-PAGE and radioactive complexes were monitored by radiography.

In vitro transcription of VDAC1, Bak, Bax, and Bax S184V yielded a product of the expected size, that upon incubation with isolated HeLa mitochondria assembled into various complexes as detected by BN-PAGE (Figure 4-7). As expected, only Bax did not efficiently integrate into mitochondria (Figure 4-7b). *In vitro* import of Bak and Bax S184V resulted in two prominent complexes of approximately 440 kDa in size (Figure 4-8a). These imported complexes were compared to the endogenous ones recognized by specific antibodies. Bax complexes detected after *in vitro* import resembled the ones found after TNFα-treatment by the Bax antibody, but were not equal. Bak *in vitro* import yielded

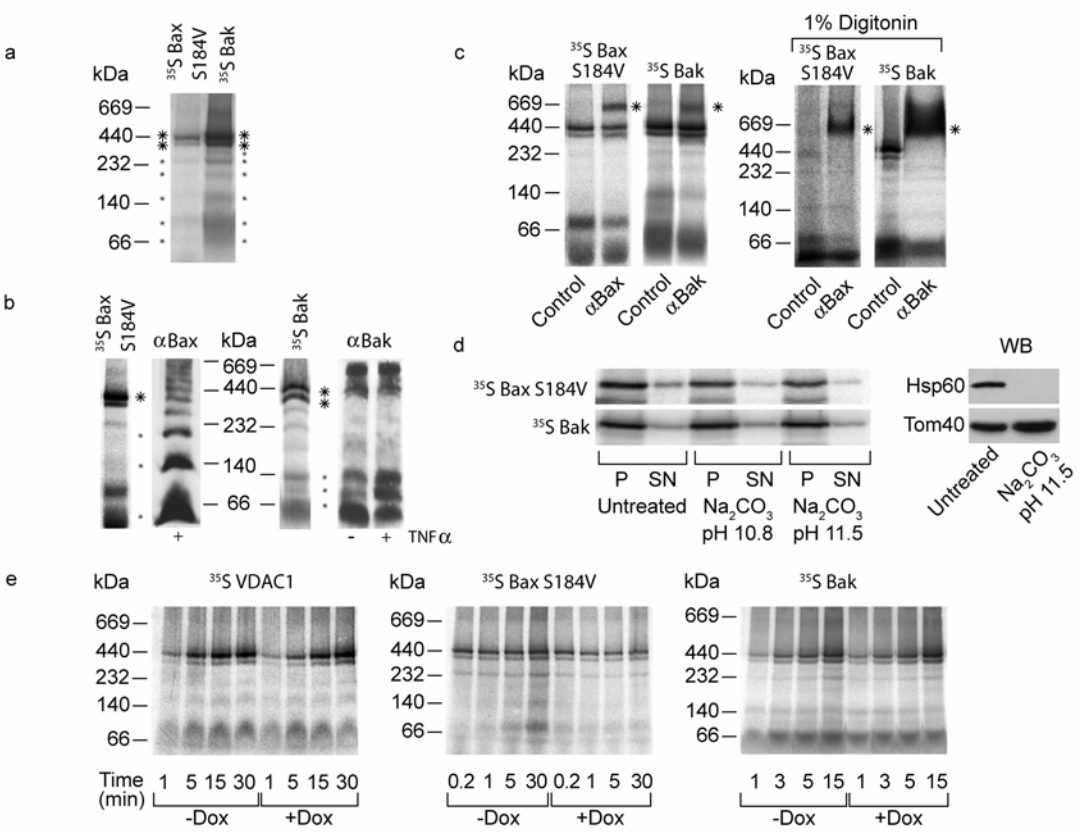

Figure 4-8. *In vitro* import of Bax, Bak and VDAC1 into mitochondria. (a) Radiolabeled lysates of Bax S184V and Bak were incubated with isolated HeLa mitochondria for 20 minutes at 37 °C. Mitochondria were lyzed in digitonin, separated by BN-PAGE and visualized by radiography. Large asterisks mark more prominent, small asterisks less prominent radioactive complexes. (b) Complexes resulting from *in vitro* import of Bax S184V and Bak as described in (a) were directly compared to endogenous complexes assessed as in Figure 4-3a for apoptotic (+TNFα) or non-apoptotic (-TNFα) HeLa cells. Complexes were marked by asterisks. (c) After *in vitro* import, mitochondria were incubated with Bax or Bak antibody, separated by BN-PAGE and subjected to radiography. Alternatively, mitochondria were solubilized in digitonin prior to antibody incubation. (d) Carbonate extraction at indicated pH values was performed following *in vitro* import of Bax S184V and Bak. Alkaline-resistant pellets (P) and TCA-precipitated supernatants (SN) were separated by SDS-PAGE and visualized by radiography. (e) *PLV-THM* cells were treated with doxycycline (Dox) for 7 days. VDAC1, Bax S184V, and Bak lysates were imported into mitochondria from control (-Dox) and Dox-treated (+Dox) cells for indicated time periods. Min, minutes.

a complex pair very similar to the one seen in the immunoblot of healthy mitochondria and some smaller less apparent complexes similar to the endogenous Bak complex pair in apoptotic mitochondria (Figure 4-8b).

It is known that *in vitro* transcription/translation of precursor proteins in rabbit reticulocyte lysate can result in unspecific products. To further investigate if the imported complexes of both proteins consist of the specific *in vitro* transcription/translation products of Bax and Bak, antibody shift experiments were performed. To this end, mitochondria were incubated with Bax or Bak antibody upon *in vitro* import of radiolabeled precursor proteins. In an additional experiment, after *in vitro* import, mitochondria were solubilized with digitonin before incubation with the antibody in order to optimize the epitope´s accessibility. In both types of experiment, a significant shift of the 440 kDa Bax S184V and Bak complexes and a reduction of some of the smaller complexes could be detected (Figure 4-8c). In samples pretreated with digitonin, this effect was even more obvious. The shift of radioactive complexes detected in this experiments upon antibody binding proves that the radioactive signal detected after *in vitro* import indeed contained Bax and Bak.

The differentiation between precursor protein that is properly integrated into mitochondria upon *in vitro* import or only mitochondrially associated is critical for the interpretation of *in vitro* import experiments. In the current study, this was achieved by the separation of complexes by BN-PAGE. To assure that the protein complexes detected on BN-PAGE represented membrane-integrated forms of Bax and Bak, mitochondria were subjected to carbonate extraction upon *in vitro* import, as Bax and Bak complexes were shown before to be resistant to this treatment (Figure 4-3b). Alkaline treatment eliminated the soluble matrix protein Hsp60 completely, but *in vitro* imported Bax S184V and Bak proteins proved to be completely resistant to the treatment (Figure 4-8d). By performing import experiments in control cell lines transduced with the empty pLV-THM vector, it was furthermore assured that the doxycycline-treatment had no influence on the import of VDAC1, Bax S184V, and Bak precursors.

Mitochondrial import of VDAC is well characterized and depends on the TOM receptors Tom20 and Tom22, the Tom40 pore and all known components of the mammalian SAM complex [Humphries, 05; Kozjak-Pavlovic, 07]. In the cell line *tom22kd-1*, VDAC1 assembly into various complexes was greatly affected under knockdown conditions (Figure 4-9a). Similarly, when all receptors were digested from the mitochondrial surface by treatment of isolated mitochondria with trypsin prior to import, VDAC1 complex assembly was nearly abolished upon *in vitro* import (Figure 4-9c). The effect of a simultaneous depletion of all TOM receptors by trypsin pretreatment of mitochondria on VDAC1 *in vitro* import is stronger compared to the effect of Tom22 depletion in the *tom22kd-2* cells, because the receptors of the TOM complex can partially substitute each other. In contrast, a lack of Tom70 receptor did not influence VDAC1 import and assembly, as described previously [Kozjak-Pavlovic, 07] (Figure 4-9b). Despite prominent defects in VDAC1 import in the absence of import receptors, no reduction in complex formation could be detected when Bax S184V precursor was incubated with the same mitochondria (Figure 4-9). Similarly, the lack of TOM receptors did not influence the assembly of Bak precursor protein in isolated mitochondria upon *in vitro* import.

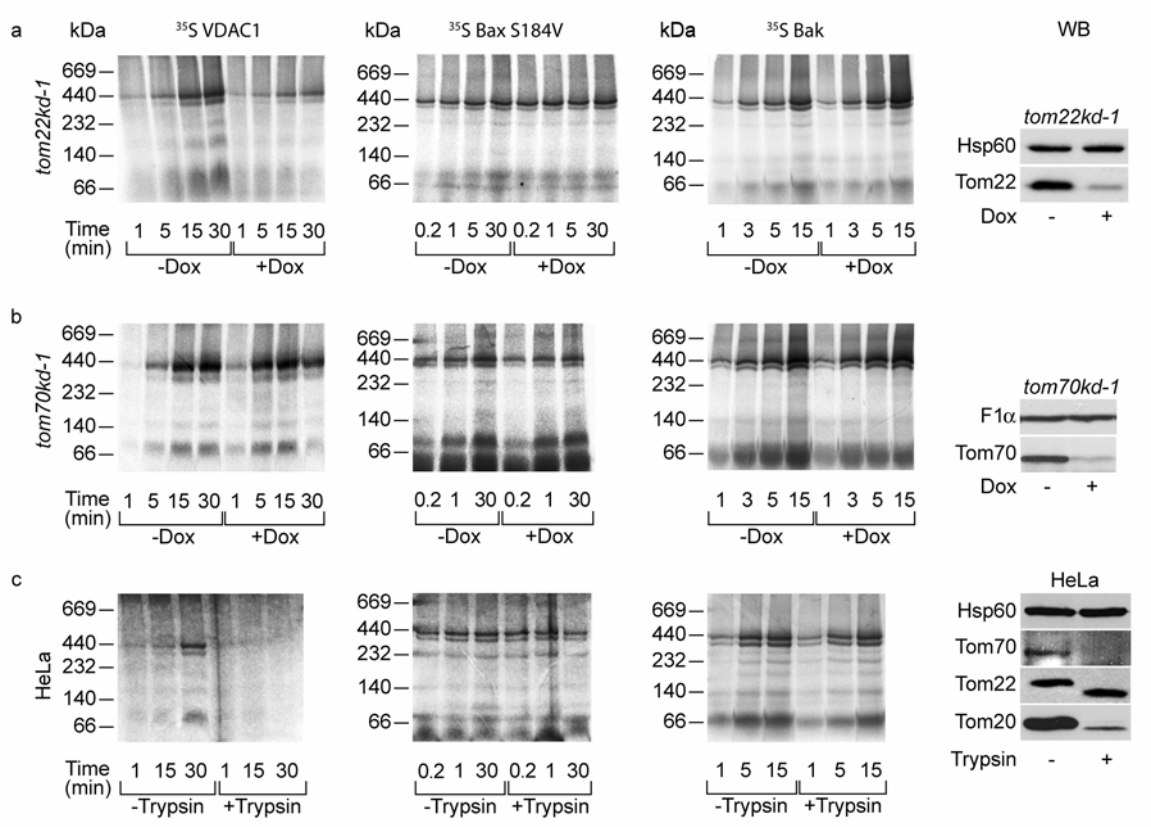

Figure 4-9. Depletion of TOM receptors does not affect complex assembly during *in vitro* import of Bax and Bak. Radiolabeled VDAC1, Bax S184V or Bak lysates were incubated for indicated periods of time with mitochondria isolated from (a) *tom22kd-1* or (b) *tom70kd-1* cells that were treated for 7 days with doxycycline (+Dox) or left untreated (-Dox). (c) Alternatively, TOM receptors on HeLa mitochondria were shaved off with trypsin prior to *in vitro* import. Mitochondria were separated by BN-PAGE, and imported complexes detected by radiography. In parallel, decrease of proteins induced by Dox (a,b) or receptor depletion (c) was controled by SDS-PAGE and western blot (WB) with antibodies specific for Tom20, Tom22 or Tom70. F1α or Hsp60 levels were used as a loading control. Min, minutes.

Finally, the impact of the Tom40 pore, Sam50, and Metaxin 2 on Bax and Bak import into mitochondria was investigated. Control experiments carried out using radiolabeled VDAC1 precursor confirmed that its import and complex assembly depends on Tom40, Sam50, and the SAM receptor Metaxin 2, and demonstrated the suitability of the used cell lines for the following experiments. To reduce unwanted secondary effects of the Tom40 depletion on other mitochondrial proteins, *tom40kd-2* cells were treated for only 5 days with doxycycline, while knockdown was induced for 7 days in the other cell lines. *In vitro* import of VDAC1 into *tom40kd-2* mitochondria was nearly abolished under knockdown conditions, proving a very efficient reduction of Tom40 in these cells (Figure 4-10a). However, lack of Tom40 had no influence on the formation of neither Bax S184V nor Bak complex formation upon *in vitro* import (Figure 4-10a). Similarly, while Sam50 depletion obviously affected VDAC1 import, the assembly of Bax S184V and Bak into their complexes in mitochondria

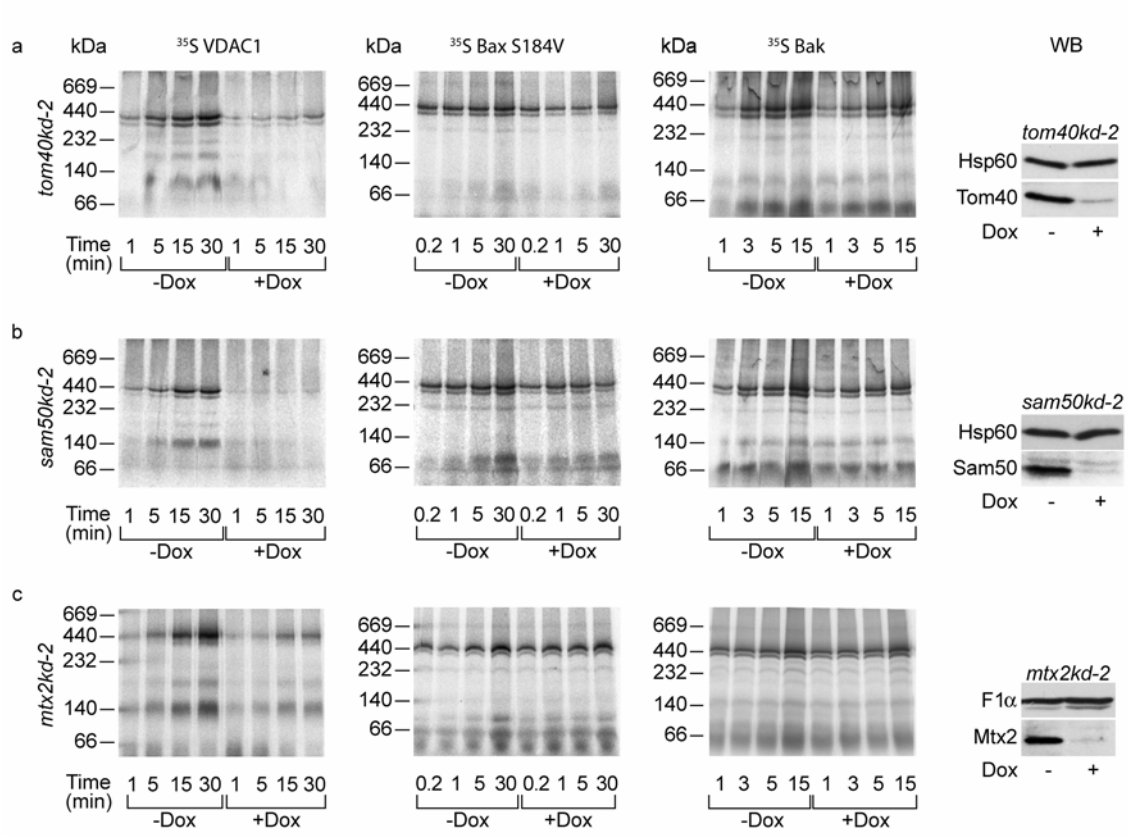

Figure 4-10. Depletion of Tom40, Sam50 or Metaxin 2 does not affect import of Bax or Bak *in vitro*. (a) *Tom40kd-2* cell were induced for 5 days, (b) *sam50kd-2* or (c) *mtx2kd-2* cells for 7d with doxycycline (Dox). Import experiments with radiolabeled VDAC1, Bax S184V and Bak were carried out as described for Figure 4-9. WB, western blot. Min., minutes.

were not influenced by a lack of Sam50 (Figure 4-10b). Also depletion of Metaxin 2 in *mtx2kd-2* cells that involves a depletion of Metaxin 1 as seen before (Figure 3-6) did not influence Bax S184V or Bak import (Figure 4-10c). In conclusion, a lack of components of the TOM or SAM complexes did not affect the mitochondrial import or complex assembly of Bax and Bak in *in vitro* experiments.

4.3 Discussion

It is well known that, during apoptosis the proapoptotic Bcl-2 proteins Bax and Bak oligomerize in the OMM. The details of the translocation and oligomerization of these proteins in the OMM, however, are still unknown. In recent reports it was found that the association of Bax with mitochondria is decreased when the TOM receptor Tom22 is sterically inhibited [Bellot, 07]. The same group later reported on a block in Bax translocation upon blocking Tom40 with a specific antibody, in accordance with another study [Cartron, 08; Ott, 07]. However, results presented in this work obtained by using a superior experimental setup contradict the earlier published data.

The HeLa cell lines with an inducible shRNA-mediated knockdown proved to be an excellent tool for the current work. The stability of the system that was achieved by a genomic integration of inducible shRNA-encoding cassettes guaranteed constant knockdown results throughout the experiments. Moreover, knockdowns of the proteins did not affect key mediators in apoptosis (Figure 4-2) that could influence the outcome of apoptosis experiments. As outlined before, HeLa mitochondria are a more valid approach for apoptosis experiments compared to yeast mitochondria, as apoptosis in yeast seems to be considerably different due to a lack of Bcl-2 proteins [Cheng, 08].

By monitoring Bax and Bak complexes after triggering apoptosis, no influence on Bax and Bak translocation into the OMM of cells that lack Tom22, Tom70, Tom40, Sam50, or Metaxin 2 could be found (Figure 4-4). In contrast to Bax, where the translocation and complex formation only occurs with the onset of apoptosis, a slow turnover rate could mask a potential dependence of Bak translocation into mitochondria on import factors. Therefore and also in order to maximize the knockdown efficiencies, the proteins targeted by shRNAs were depleted in a long-term experiment, but this did not result in a decreased assembly of Bax or Bak complexes. Only after extended Sam50 depletion an effect on Bax and Bak complex formation after TNFα-treatment was found (Figure 4-5d). As Sam50 was nearly lacking completely already after 7 days of doxycycline-treatment and therefore did not detectably decrease further after 28 days, secondary affects are more likely to have caused the small decrease in apoptotic complex assembly. In addition, levels of Bak in healthy cells do not seem to be altered by a long-term knockdown of Sam50, further questioning a role of Sam50 in Bak complex assembly.

The possibility that a protein that is decreased as a secondary effect of long-term Sam50 knockdown affects Bax translocation into mitochondria and Bax and Bak oligomerization remains open. A protein reduced upon depletion of Sam50 is VDAC. The translocation of Bak into mitochondria was shown to depend on VDAC2 in one report [Setoguchi, 06]. However, it appears unlikely that the slight reduction of Bax and Bak complexes reported here is due to a reduction of a VDAC isoform, as protein levels of VDAC on SDS-PAGE did not differ much between 7 and 28 days of Sam50 depletion, while the effect of doxycycline-treatment on Bax and Bak complex assembly was clearly stronger after the long-term treatment (Figure 4-5 and Figure 4-6). Moreover, the role of VDAC in apoptosis although suggested sometimes was questioned in several reports [Baines, 07; Cheng, 03; Shimizu, 99; Zaid, 05]. In conclusion, however, an involvement of VDAC isoforms in Bax and Bak complex assembly cannot be excluded within the present study. This question should be addressed in the future by the usage of appropriate cell lines with shRNAs targeting the VDAC isoforms. Other SAM complex substrates that decrease after Sam50 depletion and that thereby might cause secondary effects on Bax and Bak complex assembly - Tom40, Tom22, Metaxin 2, and thereby also Metaxin 1 - could be excluded in this study.

In addition to monitoring endogenous complexes of Bax and Bak, a very sensitive method, *in vitro* import of Bax and Bak was carried out. In this approach, only a small amount of radioactive precursor is used for each experiment, minimizing the risk that a saturation of an import component would result in membrane integration independent of an import factor that is normally required for a certain precursor. By the *in vitro* import of VDAC1 it was clearly shown that the knockdown induction

in the used cell lines results in the impaired import of proteins dependent on a certain import component. In the previous chapter, it was further excluded that a potential instability of the OMM would be responsible for this effect, a possibility reported earlier [Smith, 94] (see chapter 3.3.4).

The Bax S184V mutant used to model active Bax constitutively localizes to mitochondria, but might lack a possible second activation step leading to a full activation of Bax characterized by exposure of its N-terminus [Cartron, 08; Karbowski, 04; Nechushtan, 99]. This circumstances might explain the differences in complex assembly observed between *in vitro* import of Bax S184V and endogenous Bax complex formation after TNFα stimulation (Figure 4-8b). Nevertheless, as the translocation of Bax into mitochondria is of main concern in this work, the Bax S184V variant represents an appropriate model for the investigation of this issue.

In *in vitro* import studies of Bax S184V and Bak, Tom22 and Tom40 had no influence on Bax integration into the OMM , in contrast to what has been postulated before. Furthermore, the depletion of any TOM receptor or SAM complex component did not reveal any impact on Bax and Bak integration into mitochondria. An involvement of Metaxin 1 could also be excluded because this protein is depleted under knockdown conditions in *sam50kd-2* or *mtx2kd-2* cell lines (see chapter 3.3.3). In conclusion, data assessed by visualization of endogenous complexes by BN-PAGE and *in vitro* import did not indicate any dependency of Bax and Bak OMM integration and complex assembly on components of the TOM and the SAM complex.

The results presented in this work represent an important contribution to the debate on the connection between the mitochondrial import machinery and mitochondrially mediated apoptosis. The previous speculation that TOM or SAM components could build the putative MOMP pore together with Bax and Bak could be excluded in view of the data presented here. Recent publications indicate an involvement of lipids like cholesterol in the membrane integration of Bax [Christenson, 08; Lucken-Ardjomande, 08; Torrecillas, 07]. Finally, the native complexes of Bax and Bak were never presented in similar quality before. Especially the change in complex sizes in Bak after TNFα-stimulation opens new questions with regard to the components of these complexes.

5 Import of *N. gonorrhoeae* PorB into host mitochondria

5.1 A short introduction to *Neisseria gonorrhoeae*

The genus *Neisseria* comprises two important, strictly human pathogenic species, *N. meningitidis* and *N. gonorrhoeae.* The latter is the widespread cause of the sexually transmitted disease gonorrhoeae. During the initial phase of infection, gonococci typically colonize mucosal tissues of the urogenital tract. They traverse the epithelial barrier to reach subepithelial layers, where they multiply on basement membranes and provoke a local inflammation, accompanied by an infiltration of neutrophils and phagocytes. Usually, gonococcal infections remain local, but in 15 % of infected women, bacteria advance to upper genital regions and cause pelvic inflammatory disease, which is the leading cause of infertility and ectopic pregnancy. Very severe complications occur in around 1 % of gonococcal infections, where bacteria proliferate across endothelial layers in the blood and disseminate to cause a systemic infection that can lead to a colonization of joints, causing gonococcal arthritis, or sometimes to an infection of the heart or the brain, resulting in endocarditis and meningitis, respectively. Sepsis caused by systemic gonococcal infection has been described in rare cases. In newborn children, conjunctival mucosa infections can occur during birth [Harkness , 48; Hauck, 98; Merz, 00].

Various pathogenic factors on the bacterial surface are essential for the establishment of a gonococcal infection. The initiation of infection critically depends on the expression of type IV pili [Swanson, 73; Virji, 91]. They mediate adhesion to mucosal surfaces accompanied by the typical microcolony formation, consisting of 10-100 diplococci during the first hours of infection. Once *N. gonorrhoeae* overcomes the mucosal layer, pili expression is switched off by phase variation [Ilver, 98; Merz, 99]. Besides functioning in DNA transformation, pili account for the motility of gonococci that involves retraction of the pilus by its disassembly [Merz, 00; Rudel, 95; Wolfgang, 98]. This 'twitching motility' might mediate the formation of cortical plaques, characterized by an accumulation of membrane proteins like EGFR or cytoskeleton components, e.g. actin, at sites of bacterial attachment [Higashi, 07; Merz, 00]. Manipulation of host cell signaling also occurs by the pili-triggered release of Ca^{2+} from intracellular stores [Kallstrom, 98]. Importantly, through antigenic variation and phase variation, gonococci successfully escape immunosystemic recognition [Haas, 86; Meyer, 82].

Following pili-mediated attachment, tight adherence to the host and likely also invasion are mediated by the family of Opa proteins, encoded by up to 12 genes in one *N. gonorrhoeae* strain [Bhat, 92; Grassme, 96; Hauck, 03; Makino, 91]. Opa proteins are divided into two groups, Opa_{CEA} attaching to CEACAM receptors and Opa_{HS} that bind to heparan sulfate proteoglycan receptors [Chen, 95; Gray-Owen, 97]. The recognition of various receptors expressed on different tissues by Opa might reflect a cell tropism that is especially important during disseminated gonococcal infection [Merz, 00]. Depending on the combination of an Opa type interacting with a certain cellular receptor, bacterial adherence, uptake or activation of different host signaling pathways can be triggered. As an example, in phagocytic cells, Opa mediates the activation of Src-like tyrosin kinases, Rac1, PAK and JNK [Hauck, 98]. An activation of NF-κB resulting in enhanced expression of CEACAM receptor and cytokines by

Opa proteins has been documented in endothelial cells [Muenzner, 01]. Opa can also induce a rearrangement of the actin cytoskeleton that is beneficial for bacterial uptake and transcytosis of epithelial layers [Grassme, 96; Wang, 08]. Like pili, Opa proteins undergo phase variation to complicate immune responses [Bhat, 92; Stern, 84]. In addition to pili and Opa proteins, gonococcal lipooligosaccharides and porins can facilitate bacterial uptake [Bauer, 99a; Schneider, 95].

5.1.1 The major outer membrane protein PorB

Porins are transmembrane channels formed by antiparallel β-barrel sheets that mediate the passage of small hydrophilic nutrients and metabolites across the outer membrane of gram-negative bacteria, mitochondria and chloroplasts [Benz, 94]. Among *N. gonorrhoeae* porins, the major outer membrane protein PorB is essential for bacterial viability and a central virulence factor during infection [Bauer, 99a]. Two serotypes of gonococci are discriminated on the basis of the PorB allele they express: P.IB strains frequently found to cause local *N. gonorrhoeae* colonization and P.IA strains, often isolated from disseminated gonococcal infections [Sandstrom, 84]. Furthermore, P.IA expressing strains exhibit a high serum resistance *in vivo* [Cannon, 81; Judd, 87].

PorB plays a major role during invasion, as mutations in PorB or its exchange by commensal PorB species in the bacterial outer membrane abrogates entry of gonococci into epithelial cells [Bauer, 99a]. In a special experimental setup that mimics the low phosphate conditions gonococci face in the blood stream during systemic infection, P.IA, but not P.IB, mediates bacterial uptake via SREC scavenger receptors on Chang epithelial cells independent of Opa proteins [Kuhlewein, 06; Rechner, 07; van Putten, 98]. However, binding of P.IA to SREC is abrogated by competitive binding of P.IA by the heat shock protein Gp96, indicating that the prevention of uptake is beneficial for gonococci in this scenario [Rechner, 07]. PorB might also counteract bacterial clearance by the immune system, as purified PorB inhibits degranulation of neutrophils and inhibits phagosome maturation [Haines, 88; Lorenzen, 00; Mosleh, 98]. The ability of *N. gonorrhoeae* to induce apoptosis in host epithelial cells, probably to enhance traversal across the mucosal barrier, was also ascribed to PorB [Kepp, 09; Muller, 99; Muller, 00; Muller, 02]. Strains expressing the P.IA form of porin induced apoptosis more efficiently then strains harboring P.IB or PorB from a commensal strain [Muller, 99].

A unique feature of PorB is its ability to integrate into host cell membranes during the initial phase of infection, where host and bacterial membranes are in close contact [Weel, 91a; Weel, 91b]. In artificial lipids, this transfer has been shown to occur only by porins of pathogenic *N. gonorrhoeae* and *N. meningitidis* strains, but not by the commensal strain *N. sicca*. Thereby, the frequency of membrane integration was higher for the P.IA variant of porin then for P.IB [Lynch, 84]. Later, it was found that PorB can translocate from the host cytoplasmic membrane into mitochondria, where it leads to mitochondrial damage including loss of the mitochondrial inner membrane potential ($\Delta\psi$) and disruption of the mitochondrial network [Massari, 03; Muller, 00; Muller, 02]. However, as overexpression of PorB alone does not lead to apoptosis, a second trigger involving the activation of the BH3 only proteins Bim and Bmf must occur to induce cytochrome *c* release during gonococcal infection [Kepp, 09].

PorB was found to closely resemble the outer mitochondrial membrane (OMM) porin VDAC (voltage-dependent anion channel). Both porins form anion selective channels in artificial lipid membranes, where they preferentially assemble as homotrimers. They exhibit similar channel gating characteristics, as they both close at voltages higher than 40 mV or upon addition of ATP [Colombini, 79; Rudel, 96; Young, 83]. Importantly, both porins are assembled into their native membrane complexes by evolutionary conserved proteins: In neisserial outer membranes, Omp85 was shown to mediate the complex formation of PorB [Voulhoux, 04]. In eukaryotic mitochondria, the presumably Omp85-derived SAM complex assembles porins like VDAC in the OMM [Gentle, 04; Kozjak, 03; Paschen, 03; Wiedemann, 03]. Interestingly, when PorB arrives at the host cell´s mitochondria, in analogy to VDAC, it is recognized by the mitochondrial general import pore of the TOM complex, Tom40, and translocated across the OMM [Muller, 02].

Considering that the endosymbiotic mitochondrial ancestor stems from a gram-negative proteobacterium that could have been also an ancestor of *Neisseria*, the above described similarity between VDAC and PorB strongly suggests an evolutionary relationship and gives rise to the assumption that PorB, upon translocation into the host cell, is able to follow the way of VDAC into the OMM. This hypothesis was tested as described in the following and led to a surprising outcome.

5.2 Results

5.2.1 PorB affects mitochondrial integrity

Initially, the effects of PorB on mitochondria from epithelial cells were studied. For all experiments in this survey, the PorB isoform P.IA was used. Upon infection of HeLa cells with the neisserial strain N920, a derivate of the MS11 strain expressing P.IA, cells were stained with the membrane potential sensitive dye MitoTracker Orange and, upon fixation, mitochondria were labeled with a Tom20 antibody. As reported before [Muller, 00], mitochondria of infected cells revealed signs of early apoptosis, they lost their inner membrane potential and appeared largely fragmented (Figure 5-1a). When PorB amino (N)-terminally linked to the FLAG-tag was transiently expressed in Hela cells, similarly, depletion of $\Delta\psi$ was detected (Figure 5-1b). Further, cells costained for the FLAG epitope and Tom20 as a mitochondrial marker exhibited a considerable fragmentation of the mitochondrial network.

Activation of the mitochondrial apoptosis pathway usually involves a rearrangement of cristae membranes and the condensation of the mitochondrial matrix [Gottlieb, 03]. In accordance with that, infection with *N. gonorrhoeae* leads to a matrix condensation and to the loss of cristae structures [Kozjac-Pavlovic, in preparation]. It therefore was tested whether overexpression of PorB would yield similar effects. Immunogold labeling of PorB-transfected cells enabled the localization of PorB in mitochondria as verified by colabeling of PorB with Tom20. Significantly, 68 % of PorB-expressing cells showed a condensed mitochondrial matrix, indicated by its extremely electron-dense appearance in transmission electron microscopy. Moreover, the cristae structure in these mitochondria was nearly lost (Figure 5-2). These data show that transient expression of PorB, though not resulting in apoptosis, affects mitochondria in a similar way as infection with *N. gonorrhoeae* does.

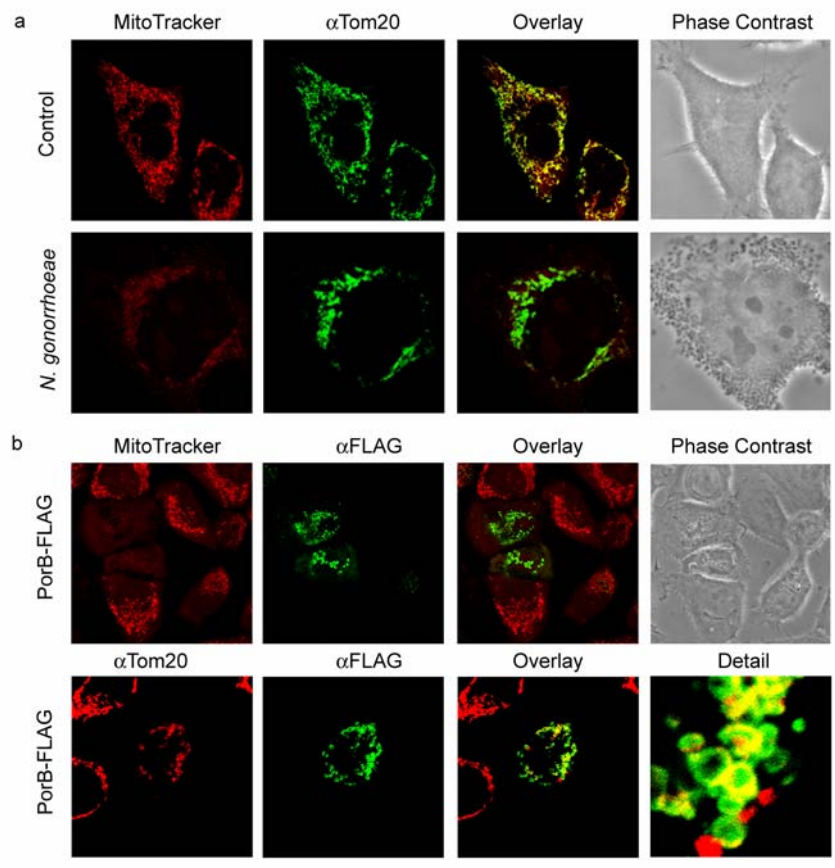

Figure 5-1. *N. gonorrhoeae* infection or transient PorB expression induces fragmentation of mitochondria and loss of the inner membrane potential. (a) HeLa cells were infected with N920 at MOI 10. After 8 hours, infected and control cells were stained with 150 nM MitoTracker Orange, fixed with PFA and stained with a primary Tom20 antibody and a Cy2-labeled secondary antibody. (b) HeLa cells were transiently transfected with PorB-FLAG. 24 h post transfection, cells were stained with MitoTracker Orange, fixed and labeled with FLAG primary and Cy2-coupled secondary antibody. Alternatively, cells were stained for FLAG and Tom20.

5.2.2 Mitochondrial import of PorB is dependent on Tom40, but not on Sam50

It was previously reported that the mitochondrial translocation of PorB depends on the Tom40 pore of the protein translocation machinery [Muller, 02]. Yet, a participation of the at that time unknown SAM complex on PorB import was not addressed in that report. Here, the import pathway of PorB was investigated by an immunofluorescence based approach. To this end, a reduction of the mitochondrial import receptors Tom20, Tom22, and Tom70, of the Tom40 pore and of the core SAM complex component Sam50 was achieved by siRNA-transfection. Knockdowns were successfully induced with a high efficiency, as controled by quantitative real-time PCR (qRT-PCR). In addition, immunoblots were carried out as far as the respective antibodies were available, confirming the high knockdown rates detected by qRT-PCR for Tom20, Tom22, and Tom40 (Figure 5-3).

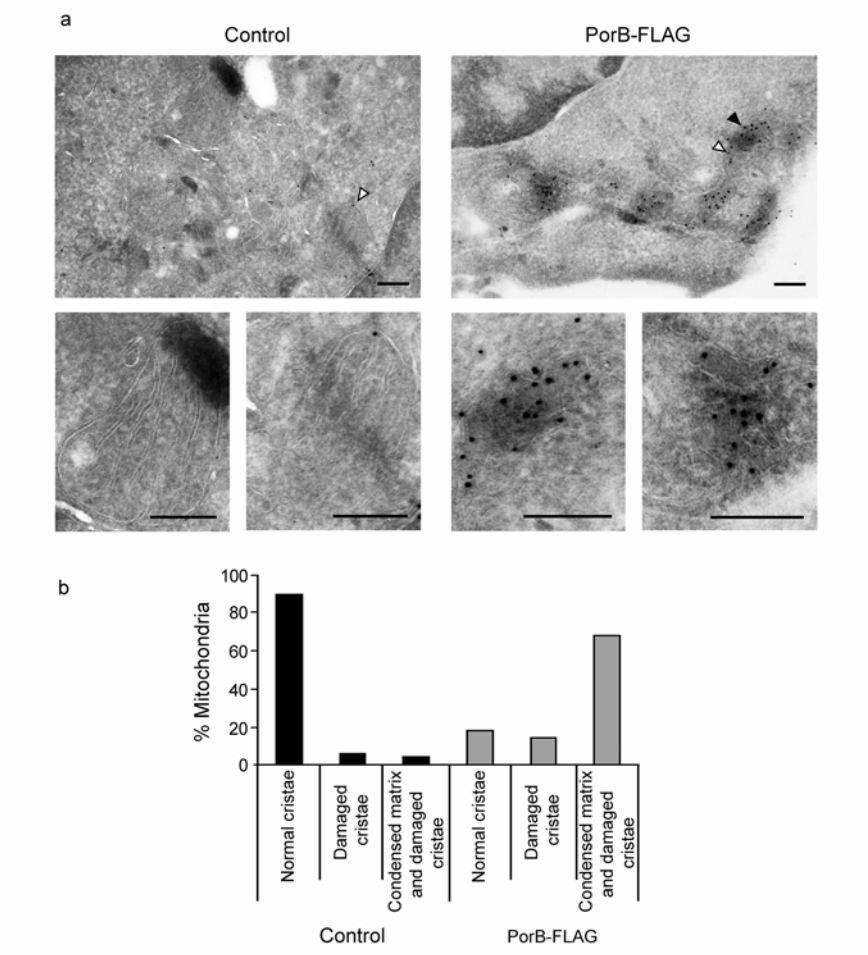

Figure 5-2. Transient expression of PorB induces condensation of the mitochondrial matrix and affects cristae structure. (a) HeLa cells were transfected with PorB-FLAG. Upon 15 hours of expression, PorB was detected by immunogold labeling with an antibody against the FLAG-tag and a secondary antibody linked to 12 nm gold particles. Endogenous Tom20 was labeled with the respective antibody and a secondary antibody linked to 6 nm gold particles. White arrows mark 6 nm, black arrows 12 nm gold particles. Scale bar, 200 nm. (b) Mitochondria were quantified according to the appearance of their cristae and matrix as indicated.

In the following series of experiments, HeLa cells with an siRNA-induced knockdown of mitochondrial proteins were transfected with PorB-FLAG. Proper mitochondrial targeting of PorB was assessed by detecting the FLAG epitope and by labeling mitochondria with the membrane sensitive dye MitoTracker Orange. In control cells that were transfected with an siRNA against the luciferase (luc) gene, PorB localized to mitochondria as seen by the typical punctuate staining pattern of the FLAG-tag. Simultaneously, $\Delta\psi$ of transfected cells was disrupted (Figure 5-4a). A similar picture was obtained when PorB was expressed in cells with a Tom20 or Tom70 knockdown (Figure 5-4b and d). In contrast, when Tom22 was depleted, many transfected cells retained their $\Delta\psi$, and PorB appeared to aggregate close to mitochondria (Figure 5-4c), indicating that PorB did not reach its usual mitochondrial place under these conditions.

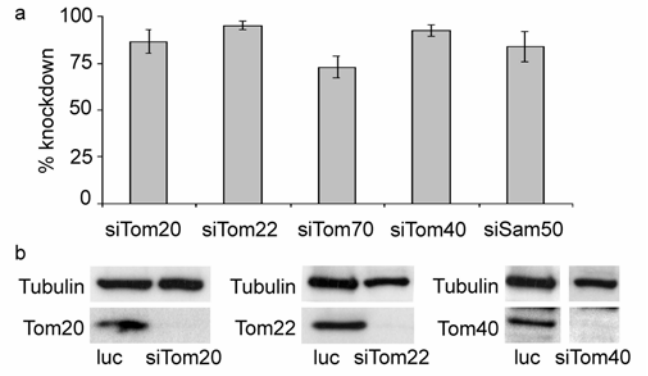

Figure 5-3. SiRNA-mediated knockdown of mitochondrial proteins. HeLa cells were transfected with siRNAs targeting mitochondrial protein import factors as indicated or with control siRNA (luc). (a) 72 hours post transfection, RNA was isolated, and mRNA levels of the targeted genes were assessed by quantitative real-time PCR. Levels of mRNA in control transfected cells were set to 100 %, and GAPDH was used as an internal standard. Results are presented from three different transfection experiments. (b) SiRNA-transfected cells were harvested 72 hours post transfection in Laemmlie buffer, and knockdowns of the indicated siRNAs were assessed by SDS-PAGE and western blot with specific antibodies if available. Tubulin served as a loading control.

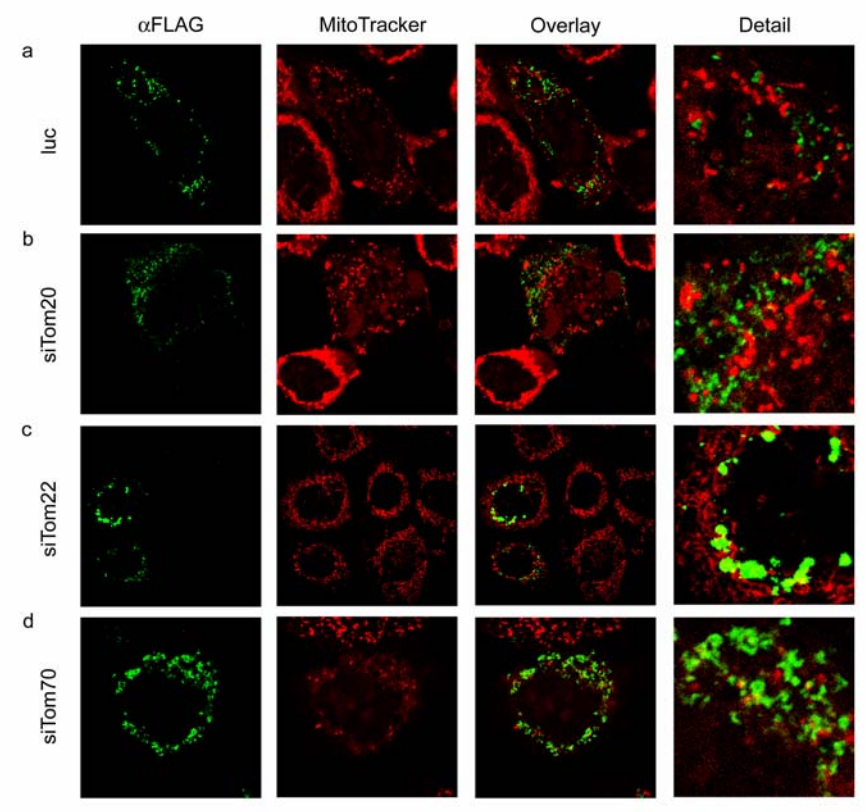

Figure 5-4. PorB import into mitochondria depends on Tom22. HeLa cells were transfected with (a) control (luc) siRNA or siRNAs targeting (b) Tom20, (c) Tom22 or (d) Tom70. 48 hours post siRNA transfection, PorB-FLAG was expressed for 24 hours. Cells were stained with 150 nM MitoTracker Orange. The FLAG-tag was labeled with the respective antibody and a secondary Cy2-coupled antibody. Cells were analyzed by confocal microscopy.

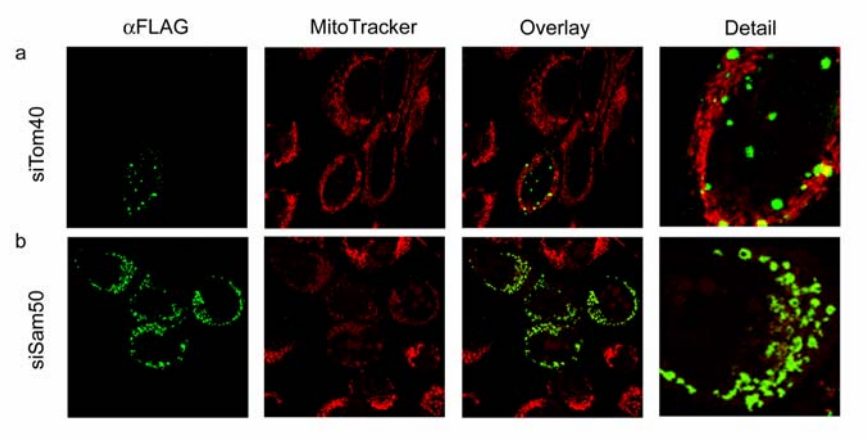

Figure 5-5. PorB import into mitochondria depends on Tom40, but not on Sam50. HeLa cells were transfected with siRNA targeting (a) Tom40 or (b) Sam50. 48 hours post transfection, PorB-FLAG was expressed for 24 hours, and cells were stained as described for Figure 5-4 and analyzed by confocal microscopy.

When the same experiment was carried out in HeLa cells transfected with Tom40 siRNA, a similar phenotype as for Tom22 knockdown could be seen, confirming the previous finding that Tom40 mediates the transfer of PorB into mitochondria (Figure 5-5a) [Muller, 02]. To assess whether the SAM complex, in analogy to the assembly pathway of VDAC, would insert PorB into the OMM, HeLa cells with a knockdown of Sam50 were transfected with the PorB-FLAG construct. Very surprisingly, PorB translocated into mitochondria unhampered by the Sam50 depletion and destroyed Δψ as in control cells (Figure 5-5b).

5.2.3 PorB is localized in the inner mitochondrial membrane

The surprising finding that PorB import and Δψ depletion occurs in the absence of Sam50 led to the idea that PorB might insert into the inner mitochondrial membrane (IMM) to execute its toxic effects. The localization of PorB was investigated by immunogold labeling of PorB-FLAG transfected cells stained for the FLAG-tag and for endogenous Tom22 or Tim23 protein as an outer or inner membrane marker, respectively. Gold particles were quantified with respect to their submitochondrial localization and grouped into three categories: 'mitochondrial periphery', which includes OMM proteins and IMM proteins closely associated with the OMM, the 'mitochondrial inner compartment', referring to IMM proteins, and 'cytoplasm or unidentified', comprising false-targeted or nonspecifically labeled proteins. As PorB´s potential to disrupt the IMM cristae structure would hamper this approach, both expression time was kept to a minimum, and for quantification cells were used that only expressed moderate amounts of PorB. Strikingly, around 70 % of labeled PorB was found in the mitochondrial inner compartment, very similar to the IMM protein Tim23 (Figure 5-6). In comparison, the majority of the OMM marker Tom22 was detected at the mitochondrial periphery. Taken together, these data strongly suggest that PorB predominantely inserts into the IMM.

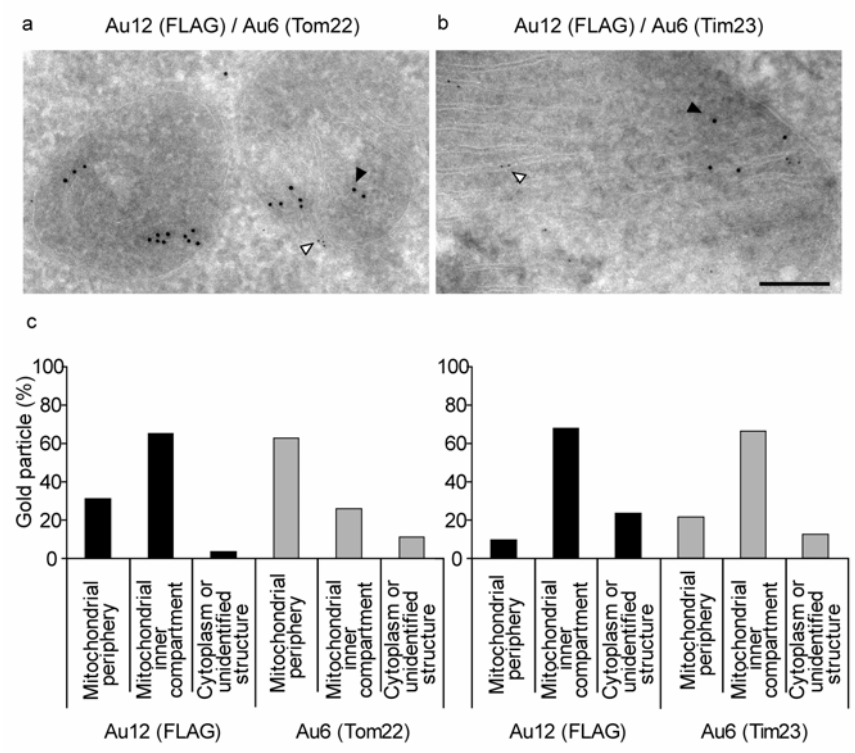

Figure 5-6. PorB is located in the inner mitochondrial membrane. PorB-FLAG was expressed in HeLa cells. 15 h post transfection, cells were colabeld with a mitochondrial marker and an antibody recognizing the FLAG-tag and a secondary antibody linked to 12 nm gold particles. (a) Endogenous Tom22 and (b) Tim23 were labeled with the respective antibodies and marked with secondary antibodies coupled to 6 nm gold particles. (c) Gold particles of the different sizes were quantified according to their intracellular localization.

5.3 Discussion

It is now widely accepted that mitochondria and today´s eubacteria derive from a common ancestor. This is also reflected by the evolutionary conservation of parts of the mitochondrial import machinery, as outlined earlier in this work (see chapter 2.3.6). Probably an interesting consequence of the eubacterial roots of mitochondria is the ability of various proteins from pathogenic bacteria to target mitochondria, suggesting that the targeting of some protein species to mitochondria has evolved already in the common ancestor of mitochondria and eubacteria. In fact, it was shown that around 5 % of the *E.coli* genes contain a classical, N-terminal mitochondrial targeting sequence [Lucattini, 04]. Quite a number of bacterial proteins were found that target host cell mitochondria, mostly affecting apoptosis signaling on the level of mitochondria [Boya, 01; Kozjak-Pavlovic, 08]. As one would expect, bacterial proteins with an N-terminal, cleavable targeting signal like for example Map from enteropathogenic *E.coli* use the mitochondrial import machinery to get into the matrix [Papatheodorou, 06]. The mitochondrial targeting of eubacterial β-barrel proteins during infection

has been described so far only for PorB from pathogenic *Neisseriae* and for Omp85 from *Acinetobacter baumanii* [Choi, 05; Massari, 03; Muller, 00].

PorB is the first pathogenicity factor with a cryptic import signal that was shown to hijack the host´s TOM complex to enter mitochondria [Muller, 02]. In this work, the earlier result of PorB translocation via Tom40 was confirmed using an immunofluorescence based approach (Figure 5-5). The same result was obtained in an *in vitro* import assay using radiolabeled PorB and the shRNA cell lines generated in this work [Kozjak-Pavlovic, submitted]. In contrast to the earlier work carried out by Müller *et al.* [02], the import receptor Tom22 instead of Tom20 was found in the current study to be crucial for PorB mitochondrial translocation (Figure 5-4). A reason for this discrepancy could be that yeast mitochondria were used in the previous study, while the present experiments were carried out in HeLa mitochondria. In order to test the hypothesis that PorB follows the import pathway of VDAC into the OMM, the possible participation of Sam50 in PorB import was investigated. Unexpectedly, the depletion of Sam50 by siRNA had no impact on the mitochondrial targeting of PorB and its ability to disrupt $\Delta\psi$ (Figure 5-5). In *in vitro* import experiments using the here described cell lines *sam50kd-2* and *mtx2kd-2*, this finding was confirmed [Kozjak-Pavlovic, submitted].

This result seems especially surprising in light of the recently published assembly pathway of PorB in *N. meningitidis* that requires the Sam50 homologue Omp85 [Voulhoux, 04]. A possible explanation for the data presented here is the divergent evolution of the sorting signal in β-barrel proteins and its recognition by the particular assembly machinery. In fact, different β-signals were reported at the carboxy (C)-termini of eubacterial and eukaryotic β-barrel proteins [Kutik, 08; Robert, 06]. While the eukaryotic signal comprises of a polar residue, a conserved glycine and two hydrophobic amino acids, the corresponding signal in *E.coli* requires a terminal phenylalanine and a stretch of hydrophobic amino acids. Even among bacteria, differences exist in the β-signal: in *Neisseria*, the penultimate amino acid usually is positively charged. This difference might hinder its recognition by Omp85 from *E.coli* and might be responsible for the toxic effect observed when expressing neisserial porins in *E. coli* [Gotschlich, 87; Robert, 06]. Although Walter *et al.* [09] reported the recognition of PhoE from *E. coli* by the eukaryotic SAM complex independent of the presence of a β-signal, such a signal with only one amino acid mismatch can actually be found in the C-terminus of this protein (Figure 5-7). In contrast, neisserial porins differ at least in two amino acids from this β-signal, providing a possible explanation for their bypass of the SAM complex. Also the recognition site of the β-signal at the Omp85 homologues seems to have diverged: in bacteria, several POTRA domains in the periplasm mediate binding to the signal, while in eukaryotes, this domain is dispensable for the assembly of β-barrel proteins [Kutik, 08; Sanchez-Pulido, 03]. The initial targeting of β-barrel proteins is independent of the β-signal [Kutik, 08]. While bacterial outer membrane proteins contain an N-terminal cleavable presequence, in eukaryotes this signal is not present, and consequently, no general targeting signal could be allocated. But, because some bacterial β-barrel proteins can target mitochondria [Walther, 09], this signal might have developed before bacteria and eukaryotes diverged, and is still conserved in some bacterial outer membrane proteins.

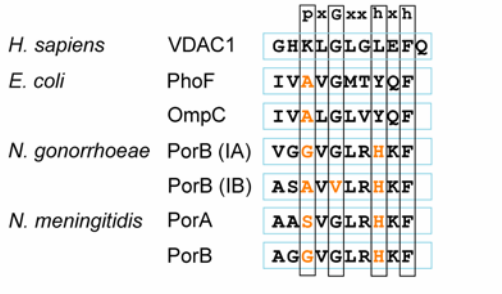

Figure 5-7. The eukaryotic β-signal in porins. The carboxy (C) -terminus of human VDAC1 is compared to the C-terminal ends of several bacterial porins. Variations of the eukaryotic β-signal are typed in orange. The β-signal consists of one large polar (p) residue, often K, H, or Q, a conserved glycine residue, and two large hydrophobic (h) amino acids, often F, L, I, V or Y. X marks arbitrary residues.

Another unexpected finding concerns the inner mitochondrial membrane localization of PorB, as was clearly seen by transmission electron microscopy using immunogold labeling and confirmed by density gradient fractionation [Kozjak-Pavlovic, submitted]. A localization of PorB in the matrix or intermembrane space cannot be excluded from the TEM study presented here, but it is shown elsewhere that following *in vitro* import, PorB is resistant to alkaline extraction, indicating that PorB is predominantly inserted into mitochondrial membranes [Kozjak-Pavlovic, submitted]. The effects of PorB on mitochondria were striking, including a loss of Δψ, cristae rearrangement, condensation of the mitochondrial matrix, and fragmentation of the mitochondrial network (Figure 5-1 and Figure 5-2). It seems realistic that these phenotypes are provoked by the formation of PorB channels in the inner mitochondrial membrane that could easily mediate the influx of protons and other small molecules into the matrix, causing Δψ depletion and matrix condensation. The rearrangement of cristae and of the mitochondrial network might be a secondary effect of Δψ loss. As outlined in the beginning, electrophysiological measurements in lipid bilayers revealed that PorB channels close at voltages higher than 40 mV, questioning an open PorB pore in the IMM with a usually higher Δψ. Recent single channel electrophysiological studies, however, revealed that ATP-induced gating of the PorB channel can affect its opening even at physiological voltages of 150 mV [Kozjak-Pavlovic, submitted].

The mechanism of PorB integration into the inner membrane is yet unknown. But, considering the often reported characteristic of PorB to spontaneously integrate into lipid bilayers, a spontaneous integration of PorB into the IMM seems possible. A similar phenomenon was published recently for *Klebsiella oxytoca*. In this organism, the secretin PulD accumulates in the bacterial plasma membrane, if the assembly pathway in the outer membrane is blocked [Guilvout, 06]. Another unknown parameter in the pathogenesis of neisserial PorB is the mode of its traversal into the host´s cytoplasmic membrane and targeting to mitochondria. To track the way of PorB in host cells will be a challenging task in the future.

6 Influence of Sam50 on mitochondrial cristae structure

6.1 A short introduction to mitochondrial membrane architecture

The textbook view of mitochondria is that of a bean shaped organelle enclosed by an outer, size constraining membrane and an inner mitochondrial membrane (IMM) with multiple invaginations, called cristae. When this structure was discovered, it was thought to simply increase the membranous surface, where respiration takes place, a widespread principle in biology [Palade , 53]. More recently, high resolution microscopy allowed more detailed insights into this highly dynamic organelle and its internal structures.

Indications for the importance of cristae structures beyond providing a site for respiration came from early studies by Hackenbrock [Hackenbrock, 66]. He discovered that cristae shape is not constant, but reflects the oxidative status of a cell by variations of the cristae volume. Later, by cryoelectron tomography, cristae shaped up as variable tubular or sometimes lamellar structures, often interconnected by various branchings [Mannella, 94]. Narrowings at the necks of cristae, called crista junctions, connect the tubules with inner membrane sections that are in close contact with the outer mitochondrial membrane (OMM), named the inner boundary membrane [Perkins, 97]. Cristae and crista junctions define additional compartments within the IMM and the intermembrane space (IMS), providing a diffusion barrier for metabolites as well as for soluble proteins and membrane proteins. The protein distribution in the IMM is asymmetric, with respiratory chain proteins located mostly in the cristae sections and a higher density of protein import complexes in the inner boundary membrane [Gilkerson, 03; Vogel, 06; Wurm, 06]; thus providing an optimized microenvironments for biochemical processes like oxidative phosphorylation or protein transport. Cristae may also limit the diffusion of ADP - a key limiting metabolite for oxidative phosphorylation - to increase its local concentration [Frey, 00; Mannella, 94]. Besides separating the IMM, cristae enclose solvents in the IMS and serve as a storehouse for proteins involved in apoptosis. It is estimated that 80 % of the mitochondrial cytochrome *c* is locked between cristae membranes, maybe to provide an additional safety barrier that prevents apoptosis in healthy cells [Scorrano, 02]. Upon induction of apoptosis and activation of the mitochondrial pathway (see chapter 4.1), crista junctions widen - possibly to facilitate the release of proapoptotic factors during mitochondrial outer membrane permeabilization - and IMS components like cytochrome *c* gain access to the IMS [Scorrano, 02]. The importance of the inner membrane topology is underlined by a variety of neurodegenerative diseases, e.g. Alzheimer´s or Parkinson´s disease, for which severe defects in cristae structure were described [Baloyannis, 06; Exner, 07; Stichel, 07].

As already indicated cristae are not static, but change their shape during different physiological conditions like changes of the nutrient levels, the osmolarity, the redox status, or in response to apoptotic stimuli. These changes affect their appearance as either predominantly tubular or lamellar, their interconnectivity, and the size of crista junctions. The modulation from one state to another is

thought of not to be a passive process, but rather to involve fusion and fission proteins in the IMM [Mannella, 08].

As in electron microscopy studies the cristae structure appears extremely regular, the question arises how this special topology is generated and maintained. It seems that under certain conditions biological membranes can fold invaginations that resemble mitochondrial cristae [Khalifat, 08; Renken, 02]. However, several proteins were found to be necessary for a proper IMM folding. In this respect subunits *e* and *g* of the ATPase complex are best characterized. In their absence, inner membranes of mitochondria form onion-like structures [Arnold, 98; Paumard, 02]. This defect is not due to a decreased respiration, as the ATPase subunits *e* and *g* are not involved in respiration, but rather trigger the dimerization of ATPase complexes. It was suggested that ATPase dimers introduce bends in the IMM at the turning points of cristae membranes [Strauss, 08]. Another protein apparently involved in shaping mitochondrial inner membranes is Opa1 [Frezza, 06]. It is localized at crista junctions and probably ties up the IMS components enclosed by cristae membranes in non-apoptotic cells. Upon depletion of Opa1 cristae seem to widen at their basis and cells are more susceptible to apoptosis. As Opa1 has an additional role in maintaining the mitochondrial network, it might link mitochondrial ultrastructure and network morphology [Frezza, 06; Griparic, 04; Olichon, 03]. Moreover, cardiolipin was also found to be involved in cristae morphology. In the absence of cardiolipin, as for example in patients suffering from Barth´s syndrome, cristae morphology is significantly altered [Acehan, 07]. Yet, it is unclear if this phenotype is caused directly by the lack of cardiolipin or rather by a lack of ATPase oligomerization that requires cardiolipin [Zhang, 02].

A highly abundant protein, whose absence causes aberrant cristae, is mitofilin [John, 05]. Occurring in two isoforms, its function is not clarified yet, but due to its high abundance, it is found in many surveys that investigate the mitochondrial proteome [Nordgaard, 08; Reifschneider, 06; Taylor, 03]. Mitofilin is an integral inner membrane protein with a large domain extruding into the IMS and was mostly found at the inner boundary membrane by immunogold labeling, where it forms high molecular weight complexes [Gieffers, 97; John, 05; Odgren, 96; Reifschneider, 06]. Interestingly, a significant reduction of the mitofilin protein level was reported in some diseases, including Down´s syndrome and Parkinson´s disease, both characterized by mitochondrial respiration defects [Arbuzova, 02; Bernert, 02; Myung, 03; Van Laar, 08]. Recently, five interaction partners of mitofilin were identified. Among these are all known components of the human SAM complex, namely Sam50, Metaxin 1 and Metaxin 2 [Xie, 07]. Remarkably, in contrast to mitofilin, these proteins are located in the OMM.

It was hypothesized that the function of this interaction might be an involvement of mitofilin in protein import [John, 05]. However, a reduction of inner membrane proteins was never reported upon depletion of SAM complex components. Considering the high abundance of mitofilin, it was suggested further that this protein provides a scaffold for mitochondrial ultrastructure and that it belts the two mitochondrial membranes together. Following this idea, the effects of Sam50 depletion on mitochondrial morphology and on the mitofilin complex were investigated.

6.2 Results

6.2.1 Sam50 depletion affects mitochondrial cristae morphology

Having the studies in mind that show mitochondrial alterations after mitofilin knockdown by RNA interference and its interaction with Sam50, it was tested by transmission electron microscopy (TEM) whether a knockdown of Sam50 reveals a similar phenotype. To this end, shRNA-expression of *sam50kd-2* cells was induced with doxycycline, and mitochondrial morphology was studied in ultra-thin sections by TEM. Strikingly, mitochondria with a Sam50 knockdown exhibited severe defects in cristae structure (Figure 6-1a). Cristae, though still visible in most cases, seemed to be detached from the inner boundary membrane and often were encircled by a second double membrane. Control cells of the same cell line revealed perfectly normal mitochondria. To exclude that a clone-specific off-target effect is responsible for the alterations of mitochondria, a cell clone with an alternative shRNA targeting Sam50 was used, *sam50kd-3*. Upon doxycycline-treatment, mitochondria in these cells were found to appear similarly altered as seen before for *sam50kd-2* cells (Figure 6-1b). To assure that the observed phenotype is not an artifact caused by doxycycline-treatment, a control cell line carrying the empty vector, named *pLV-THM*, was grown with doxycycline, and mitochondria were monitored by TEM, but revealed normal cristae morphology (Figure 6-2a). As Sam50 is an important mitochondrial protein import factor, it was questioned whether the cristae rearrangement upon Sam50 depletion is a general defect occurring when mitochondrial import is abrogated. Using a cell line with a knock-down in Tom40, mitochondria were studied as explained above. Although these cells accumulated a

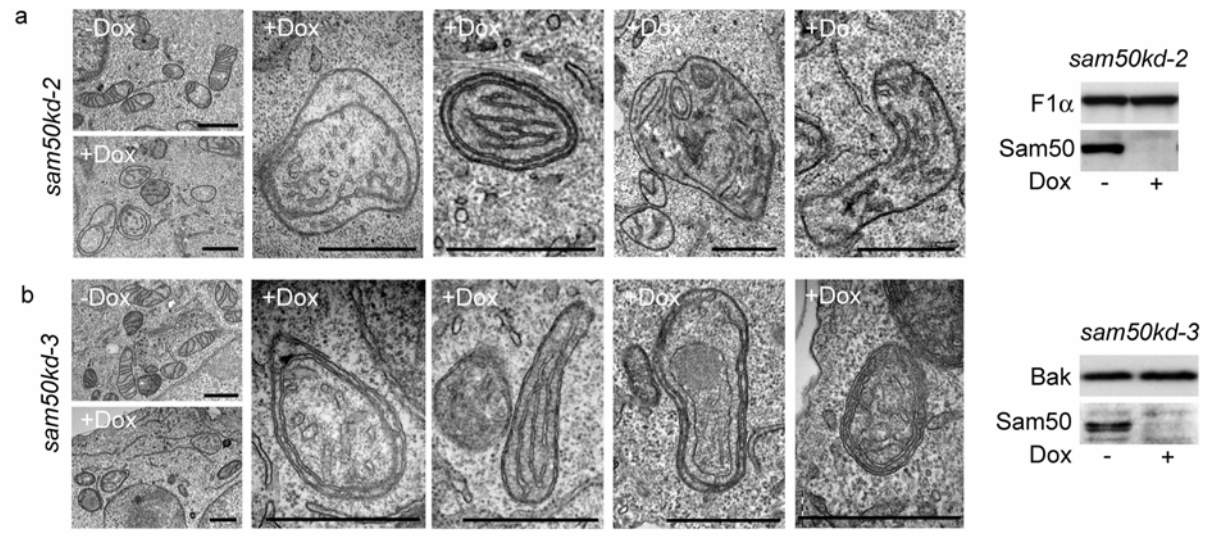

Figure 6-1. Depletion of Sam50 leads to aberrant mitochondrial cristae. (a) *Sam50kd-2* or (b) *sam50kd-3* cells were treated with doxycycline (Dox) for 7 days. Untreated (-Dox) and treated (+Dox) cells were fixed in resin, and ultrathin sections were analyzed by transmission electron microscopy. Isolated mitochondria from the same cell clones were probed for a knockdown of Sam50 by immunoblot with F1α or Bak as a loading control.

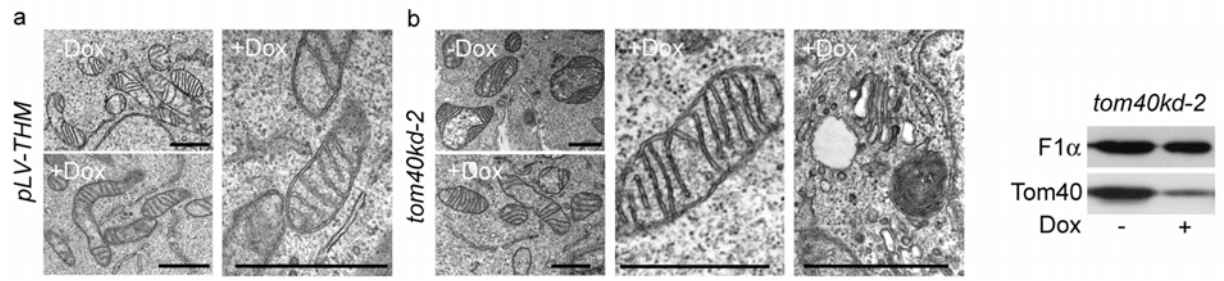

Figure 6-2. (a) *PLV-THM* cells were treated with doxycycline (Dox) for 7 days, (b) t*om40kd-2* cells for 5 days. Control (-Dox) and doxycycline-treated (+Dox) cells were analyzed by transmission electron microscopy. Isolated mitochondria of t*om40kd-2* cells were probed for a knockdown of Tom40 following 5 days Dox-induction by immunoblot with F1α as a loading control.

small amount of autophagic vesicles, mitochondria appeared relatively normal in the absence of Tom40, indicating that cristae rearrangements seen in *sam50kd-2* cells are not due to an impaired protein import in general (Figure 6-2b).

Quantification of mitochondria according to their cristae morphology revealed that 88 % of mitochondria lacking Sam50 had defects in their cristae structure, while control cells or cells with a Tom40 knockdown had only few abnormal mitochondria (Figure 6-3a). Mitochondria lacking cristae were counted extra, as it could not be assessed by TEM whether these organelles completely lacked cristae or whether they were not displayed in the ultrathin sections. By counting of damaged mitochondria, it turned out that the total number of mitochondria in 30 cells depleted of Sam50 was only 172, while in the same number of control cells 353 mitochondria were found. Therefore the question

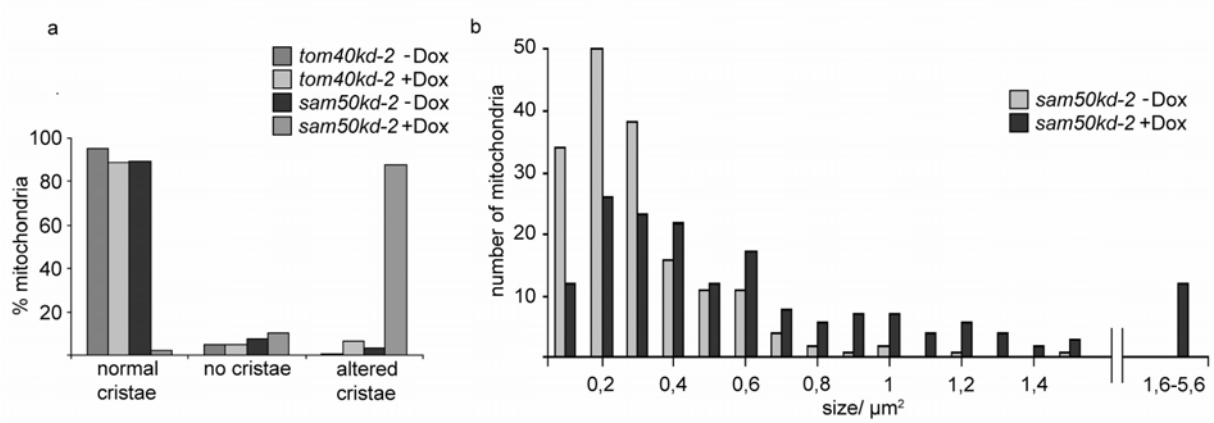

Figure 6-3. Quantification of altered mitochondria. (a) Mitochondria in *tom40kd-2* or *sam50kd-2* cells, both doxycycline-treated (+Dox) or left untreated (-Dox) were quantified according to their appearance. (b) The size of mitochondria depleted of Sam50 (*sam50kd-2* +Dox) or control cells (*sam50kd-2* -Dox) was determined using the Image J software.

arose if this reduction in mitochondrial number was due to an enlargement of mitochondria or due to their degradation, possibly by mitophagy. To address this issue, the size of individual mitochondria was determined. In *sam50kd-2* control cells that were not induced with doxycycline, the average mitochondrial size was 0.3 (±0.2) μm^2, while in cells where Sam50 knockdown was induced, an average size of 0.7 (±0.7) μm^2 with a variability of 0.1 up to 5.6 μm^2 was measured (Figure 6-3b). In Tom40 knockdown cells, the mean size of mitochondria was 0.4 (± 0.3) μm^2 in both doxycycline-treated and control cells. This observed increase in mitochondrial size suggests that the depletion of Sam50 affects the balance of mitochondrial fusion/fission activity. In conclusion, Sam50 seems to function in mitochondrial cristae morphogenesis independent of its role in protein import.

6.2.2 Lack of Sam50 alters mitochondrial network morphology

To assess the role of Sam50 in mitochondrial network dynamics, *sam50kd-2* cells, after treatment with doxycycline, were stained with the membrane potential sensitive dye MitoTracker Orange, and the mitochondrial network in these cells was compared to non-induced cells of the same cell line. Cells depleted of Sam50 totally lacked the mitochondrial network (Figure 6-4a). Instead, mitochondria

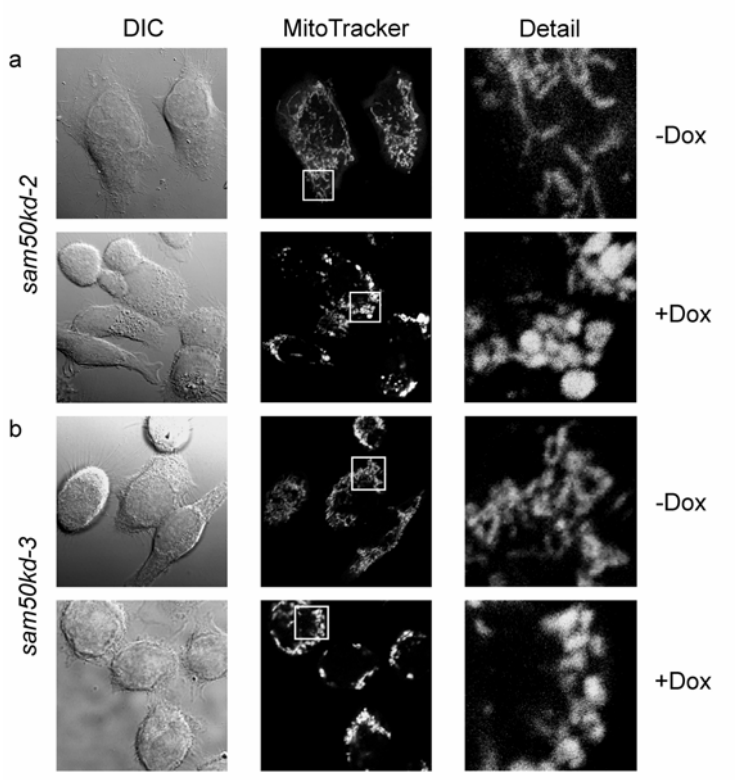

Figure 6-4. Knockdown of Sam50 affects the mitochondrial network formation. (a) *sam50kd-2* or (b) *sam50kd-3* cells were treated with doxycycline (Dox), and mitochondria from knockdown cells (+Dox) and nontreated cells (-Dox) were stained with 150 nM MitoTracker Orange. Cells were analyzed by confocal microscopy. DIC, differential interference contrast.

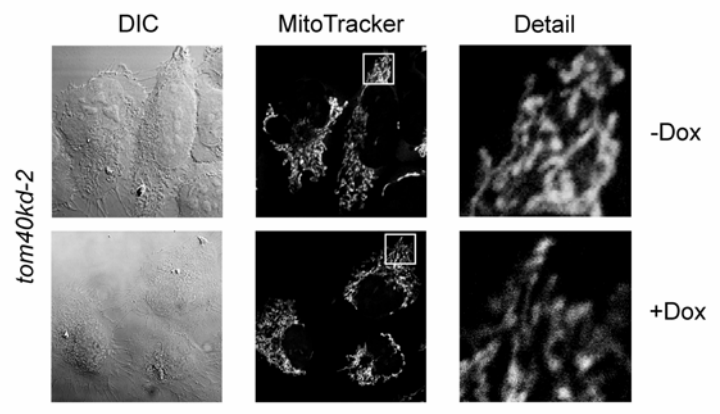

Figure 6-5. The mitochondrial network is not affected by a lack of Tom40. Untreated (-Dox) or doxycycline-treated (+Dox) *tom40kd-2* cells were stained with MitoTracker Orange and analyzed by confocal microscopy. DIC, differential interference contrast.

appeared aggregated in large spherical clumps, while they fully retained their inner membrane potential. Control cells in contrast revealed a normal mitochondrial network. A cell clone with an alternative shRNA against Sam50 similarly showed aggregated mitochondria (Figure 6-4b). To exclude a general defect in mitochondrial network formation derived from a decreased protein import, cells with a Tom40 knockdown were also analyzed. In contrast to the phenotype seen in Sam50-depleted cells, mitochondria lacking Tom40 displayed only a slight decrease in their interconnectivity (Figure 6-5). To exclude that a Sam50 knockdown decreased the levels of mitochondrial fusion proteins thereby causing the observed phenotype, the amounts of the fusion proteins Sam50, Mitofilin1, and Mitofilin2 were compared in Sam50 knockdown cells. Yet, no differences in the amount of the tested proteins could be detected between cells expressing or lacking Sam50 (Figure 6-6). Data presented here suggest that a lack of Sam50 impairs mitochondrial network formation, resulting in large spherical mitochondria.

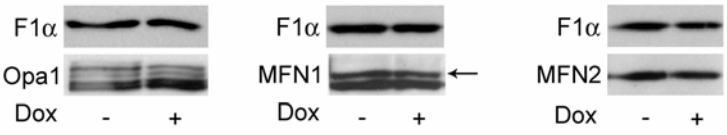

Figure 6-6. Levels of mitochondrial fusion proteins are not changed by the depletion of Sam50. Mitochondria were isolated from *sam50kd-2* cells after doxycycline (Dox)-treatment or from control cells that were left untreated. Mitochondrial proteins were separated by SDS-PAGE, and the levels of mitochondrial fusion proteins Opa1, Mitofilin1 (MFN1) and Mitofilin2 (MFN2), and of F1α as a loading control were assessed by immunoblot with the respective antibodies. The arrow marks the appropriate MFN1 band as indicated by the manufacturer.

6.2.3 Sam50 knockdown affects the mitofilin complex

By immunoprecipitation, an interaction of Sam50 with mitofilin was found previously [John, 05]. Further, the existence of a large mitofilin complex was suggested, although attempts to visualize such a complex by blue native (BN)-PAGE failed, likely due its huge size [John, 05; Reifschneider, 06; Xie, 07]. Here, in the attempt to monitor this complex in digitonin-solubilized mitochondria, similar problems were faced. A diffuse complex of around 700 kDa was detected by a specific mitofilin antibody after the separation of mitochondria by BN-PAGE (Figure 6-7a). Interestingly, in mitochondria devoid of Sam50, the amount of the 700 kDa complex increased remarkably, an effect that could even be amplified by the usage of higher digitonin concentrations. By applying the same amount of mitochondria on SDS-PAGE, equal loading of the samples on the BN-PAGE was ensured. In addition, by assessing mitofilin levels in these mitochondria, an increase of mitofilin in mitochondria from Sam50 knockdown cells was excluded (Figure 6-7b). In order to gain a better separation of the mitofilin complex, mitochondria were subjected to 2D-(BN-SDS-) PAGE. In control cells, again a complex of around 700 kDa was detected, together with a smear towards the higher molecular weight region of the gel (Figure 6-8). When Sam50 was depleted, this smear disappeared, and the 700 kDa complex species increased similarly as seen before on BN-PAGE.

In summary, a diffuse complex could be located that most likely is a solubilized part of an extremely stable mitofilin complex that cannot be dissolved by the mild conditions usually used for BN-PAGE. The stability of this complex depends on Sam50, as in the absence of Sam50, this complex falls apart into a smaller aggregate that can be displayed on BN-PAGE.

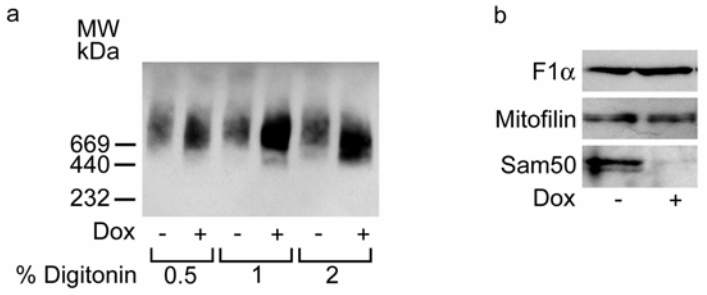

Figure 6-7. A lack of Sam50 leads to the increase of a mitofilin complex. (a) Mitochondria of *sam50kd-2* cells nontreated (-Dox) or doxycycline-treated (+Dox) were solubilized in 0.5-2 % digitonin as indicated, and separated by 3-10 % BN-PAGE. Following western blot, mitofilin complexes were detected with the respective antibody. (b) The same mitochondria as used in (a) were subjected to SDS-PAGE, and levels of Sam50, mitofilin and F1α as loading control were detected with specific antibodies.

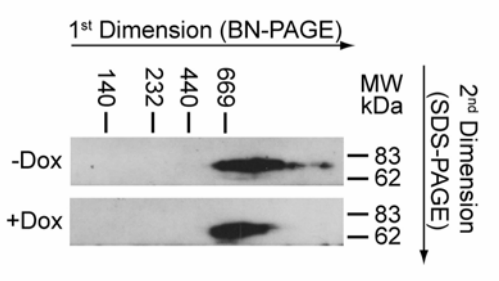

Figure 6-8. A mitofilin complex detected by 2D-(BN-SDS-) PAGE decreases in the absence of Sam50. Isolated *sam50kd-2* mitochondria solubilized in 2 % digitonin were separated by BN-PAGE as in Figure 6-7 a, and strips of the native gel were subjected to a second dimension 12.5 % SDS-PAGE. Mitofilin was detected by immunoblot.

6.3 Discussion

The intricate topology of the inner mitochondrial membrane is crucial for a proper organellar function [Mannella, 08; Zick, 09]. Though, it is not clarified yet how this structure is formed and maintained. One of few proteins that have been reported to modify the mitochondrial cristae structure when depleted by siRNA is mitofilin [John, 05]. A similar phenotype was now found by TEM in a cell line lacking Sam50, a known interaction partner of mitofilin. This observation suggests that mitofilin and Sam50 might be functionally linked in forming mitochondrial cristae architecture.

To further test this hypothesis, BN-PAGE was performed with mitochondria expressing or lacking Sam50. Due to limitations of the used Sam50 antibody, only mitofilin could be detected. Nevertheless, the mitofilin complex that accumulated upon Sam50 depletion verifies the interaction between mitofilin and Sam50 described in a recent report (Figure 6-7 and Figure 6-8)[Xie, 07]. Determining the size of the putative mitofilin complex was attempted by others previously [John, 05; Reifschneider, 06; Xie, 07]; By density gradient centrifugation, a mitofilin complex was detected in a fraction slightly smaller than the respiratory chain complex I that is 1279 kDa in size [John, 05]. A proteomic approach claimed that mitofilin forms aggregates of even up to 10 000 kDa [Reifschneider, 06]. However, more elaborated studies are necessary to better characterize this complex.

By what is known so far, mitofilin and Sam50 might function in bridging the outer and inner mitochondrial membranes. The similar phenotypes seen after Sam50 or mitofilin knockdown, disarranged cristae, and the requirement of Sam50 for the stability of a mitofilin-containing complex speaks in favour of this idea. Previously, contact sites between the OMM and the IMM were described [Knoll, 83]. Also ‘bridging particles’ were found of 10-15 nm in size that could form membrane-spanning protein complexes. The linkage of the two membranes could facilitate metabolite channeling, protein import, and stabilization of the mitochondrial ultrastructure [Mannella, 06; Nicastro, 00; Senda, 98]. Such a function was suggested already for the yeast MDM complex in the OMM in conjunction with the inner membrane proteins Mdm31 and Mdm32 [Dimmer, 05; Hobbs, 01]. In fact, very similar phenotypes on cristae morphology were shown for yeast mutants lacking one of these

factors [Burgess, 94; Dimmer, 05]. The participation of MDM in mitochondrial β-barrel protein assembly in yeast reveals another parallel to the possible function of Sam50 and mitofilin in mammalian cells described here [Meisinger, 07]. The finding that mitofilin was found to preferentially localize to the inner boundary membrane in close proximity to the OMM supports the hypothesis of a membrane-belting complex between mitofilin and Sam50 [John, 05; Odgren, 96].

It should be noted that the morphological alterations seen after shRNA-mediated depletion of Sam50 are not the same as reported for a mitofilin knockdown [John, 05]. In the absence of mitofilin, many mitochondria revealed various circular membranes, while upon Sam50 knockdown, this specific picture was only observed in few mitochondria. A possible explanation for this discrepancy might be that the functions of both proteins are not fully overlapping. For example, the complex seen on BN-PAGE of approximately 700 kDa might provide additional functions in forming the tubular shapes of cristae. Another possibility might be that mitofilin introduces bends at the basis of cristae, facilitating the IMM curvature at this position. A tethering of mitofilin towards the inner boundary membrane by Sam50 might result in exclusively local bends. A lack of this function could explain the concentric membranes seen by John *et al*. [05].

A phenotype not observed in the absence of mitofilin, but after Sam50 knockdown, is the formation of large spherical mitochondria that would point to a defect in mitochondrial fusion. Nevertheless, this is unlikely, as the mitochondria observed by TEM even had increased mitochondria, and the levels of the known proteins participating in mitochondrial fusion were not altered by a lack of Sam50 (Figure 6-4 and Figure 6-6). It seems possible that the fusion and fission apparatus in mitochondria with such an altered cristae content faces difficulties especially during fusion of the IMM, and that this may lead in some way to the observed spherical mitochondria increased in size as a secondary effect of cristae alteration. Similar spherical mitochondria were observed in yeast mutants deficient of Tom40, Sam50, Mdm10, Mmm1 and Mmm2 [Burgess, 94; Meisinger, 04; Meisinger, 07; Youngman, 04]. Although the Tom40 mutant used in the present study displayed normal mitochondria, the frequency in which this phenotype occurs more speaks in favor of a secondary effect.

The study presented here shows that Sam50 contributes to a system that scaffolds the membrane structures of mitochondria. In future, it will be exciting to explore if Sam50 is part of a hypothetical ‘mitoskeleton’ that might determine the architecture of mitochondria.

7 Conclusion and Outlook

In this thesis, three projects were discussed that are related to each other, as all three deal with protein import in mammalian mitochondria. By the establishment of cell lines that carry inducible shRNAs targeting different protein import factors, a useful tool was generated. Thereby, it became possible to investigate the role of mitochondrial protein import in a certain aspect of apoptosis, the translocation and oligomerization of Bax and Bak into mitochondria. Furthermore, the here obtained cells were used to track the way of the *N. gonorrhoeae* pathogenicity factor PorB inside host cell mitochondria [Kozjak-Pavlovic, submitted]. Results obtained using these cells were confirmed with siRNAs within the present work. Finally, on the basis of this tool, a novel role of the β-barrel protein sorting and assembly device Sam50 in the formation of mitochondrial cristae topology was found. The aim of this last chapter is to summarize the results presented before, to draw final conclusions, and to provide an outlook on future perspectives.

Protein import in yeast mitochondria was studied intensively during the past 30 years, and many fascinating details are now known regarding the complex processes required to provide mitochondria with nuclear-encoded proteins. Significantly less attention was dedicated to the homologous machineries in mammalian mitochondria, which, though being similar, exhibit important differences to their yeast counterparts. The reason for the preferential investigation of yeast is the widespread usage of conditional or temperature-sensitive yeast mutants, a system that is not available for mammalian mitochondria. By the set of cell lines generated here, a basis is provided that enables future research on the mammalian translocase systems. However, the cell lines are not fully equal to the yeast mutants. Firstly, they represent knockdowns rather than knockouts of the target genes. As a consequence, protein import of some precursors might only be slowed down, but not fully abrogated. This fact requires a monitoring of the import process kinetics rather than of one single time point. Another difference between the shRNA cell lines and temperature-sensitive yeast mutants is the onset of protein depletion: in the yeast mutants, the protein of interest is not functional from the moment of induction, while in shRNA cell lines, protein expression is only abrogated after several days of knockdown induction. The probably gradual shutdown of proteins depending on their half life and shRNA efficiency entails the reduction of its substrate proteins, that finally might influence the outcome of an experiment as a secondary effect. The parallel investigation of protein import factors that influence each other, a minimization of the duration of knockdown induction and the thorough investigation of secondary effects of a protein knockdown are crucial to circumvent this problem. Also disadvantageous is the expression of dsRed and GFP in the shRNA cell lines due to the dual vector system used. However, it was observed that the dsRed expression is very weak, allowing the detection of dyes with fluorescence emission spectra in the red range, like for example MitoTracker Orange. In conclusion, the shRNA cell lines proved to be very useful for the present studies and could form the basis for future research, especially on the mammalian SAM and the TIM22 complexes, that seem to differ considerably from their yeast counterparts. A proteomic approach might be useful to find further targets of the different protein import machineries and, by applying the recently

published QUICK method, might provide new information on interaction partners of import factors [Selbach, 06].

A topic that became fashionable some time ago was the implication of protein import factors in apoptosis. Speculations about a participation of the TOM or the SAM complex in apoptosis could be found in several research papers, followed by papers with data that supported this idea [Bellot, 07; Ott, 07]. But because of before outlined reasons, these reports were not fully convincing and mutually inconsistent. Experiments that prove TOM and SAM- independent translocation and oligomerization of Bax and Bak were provided. Considering the here presented results, a participation of protein import factors in apoptosis besides an implication in Bax and Bak mitochondrial translocation cannot be excluded completely. Though one should be careful when addressing this question by applying RNA interference (RNAi) to delete a component and make conclusions from the resulting susceptibility of cells to apoptosis. Firstly, off-target effects induced by RNAi should never be underestimated, and the usage of at least three different shRNAs or siRNAs is advisable. Studies of this kind on mitochondrial import factors were published, but the usage of not more than one interfering RNA raises questions as to the solidity of the data [Bellot, 07; Guo, 04]. Also, one would expect that a lack of such a central mitochondrial gate like Tom40 or its receptors would affect the mitochondrial concentration of all kinds of proteins involved in antiapoptotic or prosurvival signaling, making it impossible to assign an observed effect to the knockdown of Tom40 itself or to the resulting decrease of one certain protein in mitochondria.

Besides, the detection of Bax and Bak by BN-PAGE allowed the unique display of their complexes that before often were monitored by crosslinking of these proteins, yielding in the visualization of interacting proteins of only up to 250 kDa in total, or by gel filtration, where proteins usually appear in overlapping fractions, impeding the discrimination between single complexes and a clear size assignment. An especially interesting outcome was the shift of the Bak complex doublet to a smaller size during apoptosis. Various possible interaction partners were suggested for Bak both in healthy cells and during apoptosis, including Mcl-1, VDAC2, Mitofusin1 and Mitofusin2 [Brooks, 07; Cheng, 03; Willis, 05]. It would be challenging to investigate whether these putative interaction partners form one of the complexes with Bak detected by BN-PAGE, or to unravel the content of the numerous Bax complexes.

With the finding of a β-barrel protein in the inner mitochondrial membrane (IMM), as described here for PorB from *Neisseria gonorrhoeae*, the dogma was broken that porins localize exclusively in the outer membranes of bacteria and mitochondria. Only one exception managed its way into scientific journals so far, the Porin 3HL, found in the cytoplasmic membrane of B-lymphocyte cell membranes [Thinnes, 07]. In the case of PorB, the reason for its integration into the inner mitochondrial membrane is a bypass of the mitochondrial SAM complex. Surprisingly, despite being highly conserved, bacterial and eukaryotic β-barrel assembly devices recognize different C-terminal β-barrel signals. Therefore, it would be extremely interesting to address if a manipulation of the C-terminus of PorB would render it to be recognized by the eukaryotic SAM complex and prevent its insertion into the IMM as well as its toxic effects on mitochondria. As in bacterial porins, the β-signal recognized by the respective Omp85 homologue even diverges among porins from *Neisseria* species and *E. coli*,

similar studies could clarify if the exchange of the penultimate amino acid in gonococcal PorB would render it compatible with the Omp85 from *E.coli* and abrogate its toxicity when expressed in *E. coli*. Also an interesting question is whether the meningococcal PorB homologue, that was described as antiapoptotic for mammalian cells, would localize in the IMM similarly to the gonococcal porin [Massari, 03]. Finally, the possible correspondence of apoptosis induced by *N. gonorrhoeae* and the often described 'shedding' of epithelial cells in infected tissues would be worth to study [Merz, 00].

The shape of mitochondrial cristae is an old issue that received increasing attention by more recent findings of proteins that affect its morphology. Especially the rearrangement of cristae in many pathological scenarios like the diseases Alzheimer or Parkinson that become more and more important in an aging society raise interest in the molecular details of cristae formation. The here presented study only sets a starting point for the investigation of the role of Sam50 and mitofilin in shaping mitochondrial cristae. A recent finding that mitofilin interacts with Pink1, a protein implicated in Parkinson´s disease, provides a possible link between this disease and the lack of cristae found in patients suffering from it [Weihofen, 09]. Pink1 was furthermore found to be involved in mitochondrial trafficking along microtubules. It would be intriguing to investigate a putative relation between the Pink1 - mitofilin interaction and the aggregated mitochondria found after a Sam50 knockdown probably a result of impaired mitochondrial distribution. A recently reported interaction of mitofilin with mitochondrial DNA nucleoids and a connection between mitochondrial DNA and cristae structure in yeast strengthens the idea of a mitoskeleton that organizes mitochondrial substructure and awaits further exploration [Dimmer, 05; Hanekamp, 02; Wang, 06].

8 Material

Cell lines

Name	Description
HeLa	Human cervix adenocarcinoma, ATCC CCL-2
293T/17	Derived from the immortalized kidney cell line HEK 293T, ATCC CRL-11268
HeLa-KRAB	HeLa clone stably transduced with pLV-tTRKRAB-Red by Alexander Karlas

Bacterial strains

Species	Strain	Description
E. coli	DH5α	F'/endAl hsdR17 (r_K^- m_K^+) sup E44 thi-1 recA1 gyrA (Nal^R) relA1 (lacZYA-argF) U169 (80dlac(lacZ) M15)
N. gonorrhoeae	N920	MS11 P^+ (ΔpilE2) Opa^- Ngo P.1A (VP1) Cm^R,Erm^R [Bauer, 99a]

Oligonucleotides

shRNA oligonucleotides	
shTom20-1	5´-ACCACGCGTCCCCGCTCACTTTCCCTCCATTTGTTTCAAGAGAACAA ATGGAGGGAAAGTGAGCTTTTTGGAAAT-3´
	5´-TATCGATTTCCAAAAAGCTCACTTTCCCTCCATTTGTTCTCTTGAAAC AAATGGAGGGAAAGTGAGCGGGGA-3´
shTom20-3	5´-ACCACGCGTCCCCGCTTCTTTGGGCAAAGATATATTCAAGAGATATAT CTTTGCCCAAAGAAGCTTTTTGGAAAT-3´
	5´-TATCGATTTCCAAAAAGCTTCTTTGGGCAAAGATATATCTCTTGAATAT ATCTTTGCCCAAAGAAGCGGGGA-3´
shTom22-1	5´-ACCACGCGTCCCCGGATTGGGACCACTTCCTTTATTCAAGAGATAAA GGAAGTGGTCCCAATCCTTTTTGGAAAT-3´
	5´-TATCGATTTCCAAAAAGGATTGGGACCACTTCCTTTATCTCTTGAATA AAGGAAGTGGTCCCAATCCGGGGA-3´
shTom22-2	5´-ACCACGCGTCCCCGGATTGGGTCTATGTACTTCTTTCAAGAGAAGAA GTACATAGACCCAATCCTTTTTGGAAAT-3´
	5´-TATCGATTTCCAAAAAGGATTGGGTCTATGTACTTCTTCTCTTGAAAG AAGTACATAGACCCAATCCGGGGA-3´
shTom22-3	5´-ACCACGCGTCCCCGGATTGGGTCTATGTACTTCTTTCAAGAGAAGAA GTACATAGACCCAATCCTTTTTGGAAAT-3´
	5´-TATCGATTTCCAAAAAGGATTGGGTCTATGTACTTCTTCTCTTGAAAG AAGTACATAGACCCAATCCGGGGA-3´
shTom70-1	5´-ACCACGCGTCCCCGCATGCTGTTAGCCGATAAAGTTCAAGAGACTTT ATCGGCTAACAGCATGCTTTTTGGAAAT-3´
	5´-TATCGATTTCCAAAAAGCATGCTGTTAGCCGATAAAGTCTCTTGAACT TTATCGGCTAACAGCATGCGGGGA-3´

shTom40-1	5′-ACCACGCGTCCCCGGAAATACACATTGAACAACTTTCAAGAGAAGTTGTTCAATGTGTATTTCCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGAAATACACATTGAACAACTTCTCTTGAAAGTTGTTCAATGTGTATTTCCGGGGA-3′
shTom40-2	5′-ACCACGCGTCCCCGGTTGGCAACGGTAACGTTGGTTCAAGAGACCAACGTTACCGTTGCCAACCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGTTGGCAACGGTAACGTTGGTCTCTTGAACCAACGTTACCGTTGCCAACCGGGGA-3′
shTom40-3	5′-ACCACGCGTCCCCGCATGCACGCAACATACTACCTTCAAGAGAGGTAGTATGTTGCGTGCATGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCATGCACGCAACATACTACCTCTCTTGAAGGTAGTATGTTGCGTGCATGCGGGGA-3′
shSam50-1	5′-ACCACGCGTCCCCGGTGATGACGCACTTCCAAATTTCAAGAGAATTTGGAAGTGCGTCATCACCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGTGATGACGCACTTCCAAATTCTCTTGAAATTTGGAAGTGCGTCATCACCGGGGA-3′
shSam50-2	5′-ACCACGCGTCCCCGGACATTCACTGAAATCATCTTTCAAGAGAAGATGATTTCAGTGAATGTCCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGACATTCACTGAAATCATCTTCTCTTGAAAGATGATTTCAGTGAATGTCCGGGGA-3′
shSam50-3	5′-ACCACGCGTCCCCGCGGAATGTTGGTACCCATTGTTCAAGAGACAATGGGTACCAACATTCCGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCGGAATGTTGGTACCCATTGTCTCTTGAACAATGGGTACCAACATTCCGCGGGGA-3′
shMtx1-1	5′-ACCACGCGTCCCCGCTGACCTATGCCAGATTTACTTCAAGAGAGTAAATCTGGCATAGGTCAGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCTGACCTATGCCAGATTTACTCTCTTGAAGTAAATCTGGCATAGGTCAGCGGGGA-3′
shMtx1-2	5′-ACCACGCGTCCCCGCACAACCTCTGTGCCTATTGTTCAAGAGACAATAGGCACAGAGGTTGTGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCACAACCTCTGTGCCTATTGTCTCTTGAACAATAGGCACAGAGGTTGTGCGGGGA-3′
shMtx1-3	5′-ACCACGCGTCCCCGCGCCGGAACCAGATCCTATCTTCAAGAGAGATAGGATCTGGTTCCGGCGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCGCCGGAACCAGATCCTATCTCTCTTGAAGATAGGATCTGGTTCCGGCGCGGGGA-3′
shMtx2-1	5′-ACCACGCGTCCCCGCTAGGTATGGATCTCCTTACTTCAAGAGAGTAAGGAGATCCATACCTAGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCTAGGTATGGATCTCCTTACTCTCTTGAAGTAAGGAGATCCATACCTAGCGGGGA-3′
shMtx2-2	5′-ACCACGCGTCCCCGGGAAGTCAAACGTAAGATGATTCAAGAGATCATCTTACGTTTGACTTCCCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGGAAGTCAAACGTAAGATGATCTCTTGAATCATCTTACGTTTGACTTCCCGGGGA-3′
shMtx2-3	5′-ACCACGCGTCCCCGGACCAGGTCTTAGAGGATGTTTCAAGAGAACATCCTCTAAGACCTGGTCCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGACCAGGTCTTAGAGGATGTTCTCTTGAAACATCCTCTAAGACCTGGTCCGGGGA-3′
shTim23-1	5′-ACCACGCGTCCCCACCATGGTTCTGTTCTCCTCTTTCAAGAGAAGAGGAGAACAGAACCATGGTTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAACCATGGTTCTGTTCTCCTCTTCTCTTGAAAGAGGAGAACAGAACCATGGTGGGGA-3′

shTim23-2	5′-ACCACGCGTCCCCACCACTTACTCCCAGAATTCATTCAAGAGATGAA TTCTGGGAGTAAGTGGTTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAACCACTTACTCCCAGAATTCATCTCTTGAATG AATTCTGGGAGTAAGTGGTGGGGA-3′
shTim22-1	5′-ACCACGCGTCCCCGCGTGGGAGGATTTGTCTTAGTTCAAGAGACTA AGACAAATCCTCCCACGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCGTGGGAGGATTTGTCTTAGTCTCTTGAAC TAAGACAAATCCTCCCACGCGGGGA-3′
shTim22-2	5′-ACCACGCGTCCCCGGTTTCAGAGCTGGCTTAAAGTTCAAGAGACTT TAAGCCAGCTCTGAAACCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGTTTCAGAGCTGGCTTAAAGTCTCTTGAAC TTTAAGCCAGCTCTGAAACCGGGGA-3′
shTim22-3	5′-ACCACGCGTCCCCGCAGTGAAATCCCATCCATGATTCAAGAGATCA TGGATGGGATTTCACTGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCAGTGAAATCCCATCCATGATCTCTTGAATC ATGGATGGGATTTCACTGCGGGGA-3`
shTim50-1	5′-ACCACGCGTCCCCGCTTTATCTCTGGGCAATAAGTTCAAGAGACTTA TTGCCCAGAGATAAAGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCTTTATCTCTGGGCAATAAGTCTCTTGAACT TATTGCCCAGAGATAAAGCGGGGA-3`
shTim50-2	5′-ACCACGCGTCCCCGCGCCGGACATACAAATATTTTTCAAGAGAAAAT ATTTGTATGTCCGGCGCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGCGCCGGACATACAAATATTTTCTCTTGAAAA ATATTTGTATGTCCGGCGCGGGGA-3`
shTim50-3	5′-ACCACGCGTCCCCGGAAGAGAGGATGGCACTTGGTTCAAGAGACC AAGTGCCATCCTCTCTTCCTTTTTGGAAAT-3′
	5′-TATCGATTTCCAAAAAGGAAGAGAGGATGGCACTTGGTCTCTTGAAC CAAGTGCCATCCTCTCTTCCGGGGA-3`

siRNA oligonucleotides	
siluc	5′-AACUUACGCUGAGUACUUCGA-3′
siTom20	5′-AAAGUUACCUGACCUUAAAGA-3′
siTom22	5′-TGGCACATTGATCTATCTAAA-3′
siTom70	5′-AAGACAAUAAGAAGGAAUGUU-3′
siTom40	5′-AACAACTGGTTGGCAACGGTA-3′
siSam50	5′-TCGAAAGAAATTTCTCTGTAA-3′

qRT-PCR primers	
tom20	5′-TGGGTACTGCATCTACTTCG-3′
	5′-TAGTAACTCTTCACCAAGCTG-3′
tom22	5′-CAGTCCCCGGACGAATTGC-3′
	5′-CCCCATAGTCTCTCCGACAG-3′
tom70	5′-GAAGTGGCACAAGACTGTAC-3′
	5′-AGATGGCATCAGAGGTTCAC-3′
tom40	5′-TCCAGAGCATCACGCCTTG-3′
	5′-GCACCCACGATCCAGTTGC-3′
sam50	5′-CAAGTGGACCTGATTTTGGAGG-3′
	5′-AGACGGAGCAATTTTTCACGG-3′
mtx1	5′-GTCGGTGGACCTGGATAGTCT-3′
	5′-GGCACTGTAATGACTTTCCCATC-3′

mtx2	5′-GCCTTCGTCTCCCAGATTGC-3′
	5′-AAGAGAAGCTGCATTGTCAGAAA-3′
tim23	5′-GGAAGCGGCAACAAAACCAC-3′
	5′-GTTCATACCAGTTAGCGGGAC-3′
tim22	5′-CTGGTGGGTGACAAGCGTC-3′
	5′-TTTCCATCGCCTTCTCGATCA-3′
tim50	5′-AACCCGGTGGACGAAAATGG-3′
	5′-GGCTGGTAGTACGGTTCCTG-3′
GAPDH	5′-GGTATCGTGGAAGGACTCATGAC-3′
	5′-ATGCCAGTGAGCTTCCCGTTCAG-3′

Cloning primers	
pGEM4z-Bak	5′-CCATAGAATTCACCATGGCTTCGGGGCAAGGC-3′
	5′-GCTCTCAAAGCTTTCATGATTTGAAGAATCTTCGTACC-3′
pGEM4z-Bax	5′-CCATAGAATTCACCATGGACGGGTCCGGGGAGC-3′
	5′-GCTCTCAAAGCTTTCAGCCCATCTTCTTCCAGATGGT-3′
pGEM4z-Bax S184V	5′-p-TACTCACCATCTGGAAGAAGATGGG-3′
	5′-p-TACGGCGGTGAGCACTCCC-3′

Vectors

Name	Description
pMD2.G	VSV-G envelope gene (Addgene plasmid 12259)
psPAX2	HIV gag, rev and cPPT for packaging (Addgene plasmid 12260)
pLV-THM	Lentivector for cloning of shRNAs (Addgene plasmid 12247)
pLV-tTRKRAB-red	Lentivector, encodes the KRAB repressor (Addgene plasmid 12250)
pEGFP-C2 Bak	Encodes the human Bak gene [von Haefen, 04]
pEGFP-C2 Bax	Encodes the human Bax gene [von Haefen, 04]
pGEM-4z	For *in vitro* transcription from SP6 and T7 promoters (Promega)
pGEM-4z-VDAC	Encodes human VDAC1 under the SP6 promoter [Kozjak-Pavlovic, 07]
P.IA-FLAG	pOKE1, PorB P.IA from VP1 with N-terminal FLAG in pcDNA3

Antibodies and antisera

Primary antibodies

Antibody	Species	Dilution for western blot	Company
Actin (beta, clone AC-15)	mouse	1:5000	Sigma-Aldrich
AIF	rabbit	1:1000	Cell Signaling Technology
Bak NT	rabbit	1:1000	Upstate Biotechnology
Bax NT	rabbit	1:1000	Upstate Biotechnology
Bcl-2 (clone 100)	mouse	1:500	Santa Cruz Biotechnology
Bcl-$x_{L/S}$ (S-18)	rabbit	1:500	Santa Cruz Biotechnology
Cytochrome c (7H8.2C12)	mouse	1:500	BD Biosciences

F1α	mouse	1:10000	BD Biosciences
FLAG	rabbit	1:1000	Sigma-Aldrich
Hsp60	mouse	1:2000	Stressgen Bioreagents
Mitofilin	rabbit	1:2000	Abcam
MFN1	mouse	1:1000	Abcam
MFN2	mouse	1:1000	Abcam
Mtx1	rabbit	1:500	Gift from K.Terada
Mtx1	rabbit	1:100	Generated by V. Kozjac-Pavlovic
Mtx2	rabbit	1:100	Generated by V. Kozjac-Pavlovic
Opa1 (clone 18)	mouse	1:500	BD Biosciences
Sam50	rabbit	1:100	Gift from N.J. Hoogenraad
Smac/Diablo	mouse	1:1000	Cell Signaling Technology
Tim23 (clone 32)	mouse	1:2500	BD Biosciences
Tom20 (clone 29)	mouse	1:500	BD Biosciences
Tom22 [1C9-2]	mouse	1:1000	GeneTex
Tom70	rabbit	1:125	Gift from K. Mihara
Tom40	rabbit	1:100	Gift from N.J. Hoogenraad
Tom40 (H-300)	rabbit	1:1000	Santa Cruz Biotechnology
Tubulin (alpha, clone DM1A)	mouse	1:1000	Sigma-Aldrich
VDAC	rabbit	1:1000	Abcam

Secondary antibodies

Antibody	Species	Dilution	Company
ECL™ sheep, HRP-conjugated	mouse	1:3000	GE Healthcare (Amersham)
ECL™ donkey, HRP-conjugated	rabbit	1:3000	GE Healthcare (Amersham)
Cy™2 donkey, Cy2-conjugated	mouse	1:100	Jackson ImmunoResearch
Cy™2 goat, Cy2-conjugated	rabbit	1:100	Jackson ImmunoResearch
Cy™3 donkey, Cy2-conjugated	mouse	1:100	Jackson ImmunoResearch

Enzymes

Name	Company
Phusion DNA Polymerase	Finnzymes
Expand High Fidelity^{PLUS} DNA Polymerase	Roche Applied Science
Taq-polymerase	Home made
T4 DNA Polymerase	New England BioLabs
T4 DNA Ligase	New England BioLabs
Restriction Endonucleases	New England BioLabs

Chemical reagents

Name	Company
TNFα	BD Biosciences
Cycloheximide	Sigma-Aldrich
Doxycycline	BD Biosciences
dNTPs	Roche Applied Science
Lipofectamine 2000	Invitrogen
MitoTracker Orange	Molecular Probes
Polybrene	Sigma-Aldrich
ECL plus	NEN Life Science Products
^{35}S methionine/cysteine	GE Healthcare and PerkinElmer
Digitonin	Sigma-Aldrich

All reagents not listed here were purchased from Biomol, Biorad, Fluka, Merck, Roth, Serva and Sigma-Aldrich.

Media and Supplements

Cell culture media and supplements including RPMI, DMEM, OptiMEM, L-Glutamine, Penicillin/ Streptomycin, trypsin and PBS were purchased from Invitrogen (GIBCO® Invitrogen Cell Culture). FCS was from Biochrom. For cultivation of *E. coli*, LB-Broth Base or LB-Agar from Invitrogen were prepared according to the manufacturer's instructions and supplemented with 100 µg/ml ampicillin. For growth of *N. gonorrhoeae*, GC agar base supplemented with Proteose Peptone no. 3 and 1 % vitamin mix (Difco) was used.

Kits

Name	Company
RNAifect	Qiagen
Qiafilter Plasmid Midi Kit	Qiagen
PCR Purification Kit	Qiagen
QuantiTect SYBR Green RT-PCR	Qiagen
QuantiTect RT Mix	Qiagen
Rneasy Mini Kit	Qiagen
TNT® Quick Coupled Transcription/ Translation System	Promega

Labware

Name	Company
Amersham Hyperfilm ECL	GE Healthcare
PVDF membrane	PerkinElmer Life Science
Whatman 3mm chromatography paper	Schleicher and Schüll

Falcon tubes with cell strainer	BD Biosciences
DuraporeTM 0.45 µm	Millipore
96-well PCR plates	Abgene
Absolute QPCR seal	Abgene
Imaging Plate BAS-IP MS 2325	Fujifilm Life Science
Imaging Plate Cassette BAS 2325	Fujifilm Life Science
Cell culture flasks and plates	TPP
Reagent tubes	Greiner, Sarstedt
RNase*Zap*®	Ambion
ART® tips	ThermoFisher Scientific
Cell scraper	Sarstedt
Dounce homogenizer	Kimble Kontes

Instruments

Name	Company
HERA cell 150 incubator	Heraeus
Cell culture hood HB2472	Thermo Scientific
Shaking incubator	New Brunswick
Mini-PROTEAN III cell	Biorad
PROTEAN II xi cell	Biorad
PerfectBlue Dual Gel system Twin M	Peqlab
Power supply EPS 3501XL	Amersham
Mini Trans-Blot® Electrophoretic Transfer Cell	BIORAD
PhosphoImager FLA-3000	Fujifilm Life science
Sorvall RC 5B	Du Pont
Varifuge 3.ORS	Heraeus
Centrifuge 5417 C	Eppendorf
TL-100 Ultracentrifuge	Beckmann
GeneAmp PCR system 2400	PerkinElmer
Duomax 1030	Heidolph
Gradient Mixer	H. Hölzel
Pump P-1	Pharmacia Biotech
Microscope IX50	Olympus
Confocal microscope SP1	Leica Microsystems
Confocal microscope TCS SPE	Leica Microsystems
LEO 906E transmission EM	Zeiss
Abi Prism 7900	AME Bioscience Biosystems
MoFlo® Flow Cytometer	Dako

Software

Name	Company
MS office	Microsoft
Adobe Acrobat 7.0	Adobe
Photoshop 7.0	Adobe
Illustrator CS	Adobe
Reference Manager 11	Thompson ISI research soft
BasReader 3.14	Fujifilm Life science
Aida Image analyzer 4.03	Fujifilm Life science
Vector NTI 10	Invitrogen
Leica Confocal Software	Leica
SDS2.2.2	Applied Biosystems
ImageJ	Wayne Rasband, NIH
BLOCK-iT™ RNAi Designer	Invitrogen

Buffers and Solutions

Cloning and DNA transfection	
5x Annealing buffer	500 mM KAcetate, 150 mM Hepes-KOH pH7.4, 2 mM MgAcetate
10x Taq buffer	100 mM KCl, 100 mM $(NH_4)_2SO_4$, 100 mM Tris-HCl, pH 8.5, 20 mM $MgSO_4$, 1 % Triton X-100
2x HBS	50 mM HEPES pH 7.05, 10 mM KCl, 12 mM Dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH was adjusted to 7.05 and sterile filtered (0.45 µm pore size)

Experiments with mitochondria	
SET-buffer	250 mM Sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.6
Buffer B	20 mM HEPES pH 7.6, 220 mM Mannitol, 70 mM Sucrose, 1 mM EDTA, 0.5 mM PMSF
Buffer A	Buffer B + 2 mg/ml BSA (fatty acid free)
Import buffer	3 % (w/v) fatty acid free BSA, 250 mM Sucrose, 80 mM KCl, 5 mM $MgCl_2$
Swelling buffer	10 mM HEPES pH 7.5
Control buffer	10 mM HEPES pH 7.5, 250 mM Sucrose

SDS-PAGE	
4x Laemmlie buffer	0.25 mM Tris-HCl pH 6.8, 8 % (w/v) SDS, 40 % (v/v) Glycerol, 2.8 M β-mercaptoethanol, 0.2 % (w/v) Bromphenol Blue
10x SDS running buffer	250 mM Tris-Base, 1.918 M Glycine, 1 % (w/v) SDS
12.5 % SDS running gel	12.5 % Acrylamide/Bisacrylamide 30 % (37.5:1), 375 mM Tris-HCl pH 8.8, 0.1 % (w/v) SDS, 15 µg/ml APS, 0.15 % TEMED
3 % SDS stacking gel	3 % Acrylamide/Bisacrylamide 30 % (37.5:1), 80 mM Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 20 µg/ml APS, 0.2 % TEMED

BN -PAGE	
Digitonin buffer	50 mM NaCl, 10 % Glycerol, 20 mM Tris-HCl pH 7.4, 0.1 mM EDTA Freshly added: 1 mM PMSF, 0.5-2 % (w/v) Digitonin
10x Loading buffer	5 % (w/v) Coomassie Brilliant Blue G-250, 100 mM Bis-Tris pH 7.0, 500 mM E-Amino-N-Caproic Acid
3x BN gel buffer	200 mM E-Amino-N-Caproic Acid, 150 mM Bis-Tris pH 7.0
BN running gel (lower percentage)	4 % Acrylamide/Bisacrylamide (49.5 % T, 3 % C), 1x BN gel buffer, 5 µg/ml APS, 0.04 % TEMED
BN running gel (higher percentage)	10 or 13 % Acrylamide/Bisacrylamide (49.5 % T, 3 % C), 1x BN gel buffer, 17.2 % Glycerol, 3 µg/ml APS, 0.04 % TEMED
BN stacking gel	4 % Acrylamide/Bisacrylamide (49.5 % T, 3 % C), 1x BN gel buffer, 5 µg/ml APS, 0.04 % TEMED
BN anode buffer	500 mM Bis-Tris pH 7.0
10x BN cathode buffer	500 mM Tricine, 150 mM Bis-Tris pH 7.0, 0.2 % (w/v) Coomassie Brilliant Blue G-250
10x Clear BN cathode buffer	500 mM Tricine, 150 mM Bis-Tris pH 7.0

Western blot	
Transfer buffer	2 mM Tris, 15 mM Glycine, 0.002 % (w/v) SDS, 20 % MeOH
TBS-T	10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 % Tween 20
Coomassie stainer	40 % EtOH, 7 % Glacial Acetic Acid, 0.2 % (w/v) Comassie R-250
Coomassie destainer	30 % EtOH, 10 % Glacial Acetic Acid

Immunofluorescence	
Moviol	13.3 % (w/v) Moviol 4-88, 33 % (w/v) glycerine in 0.15 M Tris-HCl pH 8.5
Blocking buffer	10 % goat serum, 0.2 % Triton X-100 in PBS
Antibody dilution solution	3 % goat serum, 0,05 % Tween 20 in PBS

9 Methods

9.1 Cultivation of eukaryotic and bacterial cells

Culture of human cells

All cells were grown at 37 °C in a moist atmosphere with 5 % CO_2 in plastic cell culture flasks. HeLa cells and HeLa-derived cell lines were grown in RPMI medium supplemented with 10 % heat-inactivated FCS and 1 % penicillin/ streptomycin. 293T cells were cultivated in DMEM medium with 10 % heat-inactivated FCS, 1 % penicillin/ streptomycin and 4 mM L-glutamine. Cells were passaged every second or third day when confluency had reached 70-90 %. Therefor, cells were washed once with PBS and overlayered with trypsin for 3 -5 minutes. After inactivation of trypsin by adding culture medium, cells were singularized by pipetting and splitted in a ratio between 1:10 and 1:4 depending on individual growth rates. After 12-18 passages, cells were replaced by thawing up fresh stocks that were stored in liquid nitrogen. Stocks were prepared by diluting 3-10 x 10^6 cells in FCS and 10 % DMSO. Apoptosis was induced by adding 25 ng/ml TNFα and 10 µg/ml cycloheximide to the cell culture medium for 4 hours. Expression of shRNA was triggered by growing cells in medium containing 1 µg/ml doxycycline for 5-7 days as indicated.

DNA transfection of HeLa cells

Around 75 000 HeLa cells were seeded in 12-well plates on the day before transfection. For transfection, 0.75 µg DNA was diluted in 50 µl OptiMem, and, in a different tube, 1.5 µl Lipofectamine 2000 was mixed with 50 µl OptiMem. Both mixtures were incubated for 5 minutes, combined and incubated for further 20 minutes at room temperature. In the meantime, medium was removed from the cells and replaced by 400 µl prewarmed OptiMem. The transfection mix was added to the cells and incubated for 4-6 hours until replaced by culture medium.

siRNA transfection of HeLa cells

Transfection of siRNA was performed using the RNAifect kit. To minimize the risk of RNAse contamination, working materials were cleaned with RNase*Zap*®, and RNAse free filter tips were used. 1 µg siRNA was mixed with 90 µl EC-R, vortexed and incubated at room temperature for 5 minutes. 6 µl RNAifect was added, the mixture was vortexed for 10 seconds and incubated for 20 minutes at room temperature. In the meantime, 200 000 HeLa cells were seeded into 12-well plates in 400 µl cell culture medium. 100 µl transfection mix was added, and cells were incubated over night for maximal 16 hours before the transfection mix was replaced by cell culture medium.

Culture of bacterial cells and infection

N. gonorrhoeae strain N920 was streaked on GC-agar and grown for 16-18 hours at 37 °C and 5 % CO_2. Before infection, neisseria were passaged once over night. HeLa cells grown in the absence of antibiotica were used exclusively for infection. The bacterial number was determined by measuring the optic density of neisseria that were taken up with a swamp at 550 nm.

9.2 Generation of stable shRNA cell lines

Production of lentivirus

Lentivirus was produced by the RNAi group. Therefor, 2 x 10^6 293T cells were seeded into 10 cm dishes. On the following day, cells were transfected by the $CaCl_2$ method with lentiviral packaging, envelope and shRNA-encoding vectors that were all prepared endotoxin-free (Qiafilter Plasmid Midi Kit). 6 µg pMD2.G, 15 µg psPAX2 and 20 µg pLV-THM were mixed, filled up to 250 µl with ddH_2O, and 0.25 M $CaCl_2$ was added. This mixture was vortexed well and added slowly dropwise to 500 µl 2x HBS, while vortexing at maximum speed. After an incubation of 30 minutes at room temperature, the precipitate was added slowly and dropwise to the 293T cells. The plate was gently shaken and incubated at 37 °C overnight. Seven hours after transfection, medium was changed. 48 hours after transfection, the cellular supernatant was collected and centrifuged at 2500 rpm for 10 minutes at 4 °C, filtered through a 0.45 µm sterile filter and directly used for the transduction of target cells.

Transduction of HeLa cells with lentivirus

Transduction was carried out by the RNAi group. 250 000 HeLa-KRAB cells were seeded in a 6-well plate one day before infection. 5 ml of virus-containing supernatant was mixed with 10 µg/ml polybrene and 10 mM HEPES, and added to the cells in 500 µl RPMI. Cells were incubated at 37 °C overnight for maximal 16 hours, then medium was replaced.

Selection of single cell clones

Transduced cells were grown for 3-4 days. Then, cells were trypsinized, and 1 x 10^6 cells were separated by a cell strainer. The remaining pool of cells was kept as a stock in liquid nitrogen. Cells were sorted with a MoFlo® flow cytometer by the flow cytometry core facility of the DRFZ. 1 cell per well was sorted into a 96-well plate in RPMI medium supplemented with 10 % FCS and 1 % penicillin/streptomycin.

9.3 Biochemical assays

Isolation of mitochondria

HeLa cells from 1-2 confluent 15 cm dishes were washed in PBS and harvested in 5 ml PBS with a rubber policeman. Cells were pelleted at 800 g for 5 minutes at 4 °C. When apoptosis experiments were performed, cells were directly scraped off in medium, pelleted by centrifugation and washed once with PBS. The pellet was resuspended in 1 ml buffer A, filled up to 4 ml with buffer A, and cells were swelled on ice for 60 minutes. The cells were then homogenized using a glass homogenizer with a teflon pestle fixed to an electric rotor by applying 35 strokes. The sample was then pelleted at 800 g for 5 minutes at 4 °C. The resulting supernatant was centrifuged at 10 000 g for 10 minutes at 4 °C, and, after washing in buffer B, the pellet was resuspended in 20-150 µl buffer B. The mitochondrial concentration was determined by diluting 1-5 µl of mitochondria in 600 µl 0.1 % (w/v) SDS and measuring the absorption at 280 and 320 nm. The concentration was calculated as follows [Clarke, 76]:

$$c_{protein} = \frac{A_{280nm} - A_{320nm}}{1.05} \cdot \frac{600\ \mu l}{x\ \mu l}\ [\mu g/\mu l]$$

x = 1-5 µl mitochondria

Isolated mitochondria were stored at -80 °C.

In vitro import of ^{35}S-labeled proteins into mitochondria

In vitro transcription and translation was carried out using the TNT® Quick Coupled Transcription/ Translation system according to the manufacturer´s instructions. 50-70 µg freshly isolated mitochondria were mixed with 100 µl import buffer supplemented with 2 mM ATP and 10 mM sodium succinate. 5 µl ^{35}S-labeled Bak or VDAC lysate was added, or in the case of Bax and Bax S184V, 15 µl radiolabeled lysate was used. The import mix was incubated at 25-37 °C for up to 1 hour. Samples were centrifuged at 10 000 g for 5 minutes at 4 °C and supernatants were removed. 500 µl cold SET-buffer was added, and samples were centrifuged again with the pellet towards the axis of the centrifuge. Pellets were stored at -80 °C until analyzed.

Digestion of isolated mitochondria with proteinase K

For the swelling control, 50 µg isolated mitochondria were resuspended in 400 µl control buffer or swelling buffer. During incubation on ice for 30 minutes, samples were pipetted up and down 10 times every 10 minutes. Then, samples were centrifuged at 10 000 g for 10 minutes at 4 °C. For proteinase K digestion, mitochondrial pellets were resuspended in 100 µl control buffer with 50 µg/ml proteinase K and digested on ice for 30 minutes with occasional pipetting. To inhibit the protease, 2 mM PMSF was added for 10 minutes, pellets were collected by centrifugation and washed once in 100 µl SET-buffer containing 2 mM PMSF at 4 °C. Mitochondria were resuspended directly in 15 µl Laemmlie buffer after a final centrifugation step.

Digestion of mitochondrial surface proteins by trypsin

Receptor shaving was carried out by incubating 100 µg mitochondria with 20 µg/ml trypsin or with 600 µg/ml soybean trypsin inhibitor as a control in 100 µl SET-buffer for 10 minutes on ice. The enzymatic reaction was stopped by adding 600 µg/ml soybean trypsin inhibitor, and after 10 minutes on ice, mitochondria were washed once with 1 mg/ml soybean trypsin inhibitor in 1 ml SET-buffer.

Alkaline extraction of mitochondria

50 µg isolated mitochondria were mixed with 100 mM icecold Na_2CO_3 (pH 10.8 or 11.5), incubated on ice for 30 minutes and pipetted up and down 10 times every 10 minutes. Samples were then centrifuged at 100 000 rpm for 30 minutes at 4 °C. Pellets were resuspended in Laemmlie buffer, and supernatants were precipitated with TCA.

TCA-precipitation

1 ml protein solution was mixed with 250 µl 72 % TCA and 10 µl 1.25 % sodium deoxycholat. After 30 minutes incubation on ice, the sample was centrifuged at 14 000 rpm for 30 minutes at 4 °C. The pellet was washed twice in -20 °C cold acetone at 14 000 rpm for 10 minutes at 4 °C. Pellets were then dried for approximately 10 minutes at room temperature and resuspended in Laemmlie buffer. In samples where the pH was too low, indicated by a yellow color of the added Laemmlie buffer, 1-2 µl 1 M NaOH was added.

Antibody shift of mitochondrial protein complexes

Antibody shift after *in vitro* import was carried out either without or after lysis in digitonin. For digitonin lysis, 50 µg mitochondria were resuspended in 50 µl digitonin buffer containing 1 % digitonin and 1 mM PMSF, and incubated for 10 minutes on ice. Samples were centrifuged at 16 000 g for 10 minutes at 4 °C, and supernatants were moved to a fresh tube before 20 µl Bak or 10 µl Bax antibody was added. For antibody shift without digitonin pretreatment, 50 µg pelleted mitochondria after *in vitro* import were resuspended in 50 µl import buffer and antibody was added. Samples were incubated for 1 hour on ice with gentle shaking every 20 minutes. Samples not yet treated with digitonin were solubilized in detergent, while digitonin-pretreated samples upon addition of sample buffer were directly applied on BN-PAGE.

SDS-PAGE

Samples were lysed in Laemmlie buffer and denatured additionally at 95 °C for 5 minutes. Then, 10-20 µl of the sample was applied on a discontinuous resolving 12.5 % SDS-gel with a 3 % stacking gel in a Mini Protean III chamber. The run was performed in 1x SDS running buffer, initially at 60 V until the samples had reached the resolving gel, then the voltage was raised to 120 V.

BN-PAGE

50-100 µg mitochondria were pelleted and resuspended in 40 µl ice-cold digitonin buffer with 0.5-2 % digitonin and 1 mM PMSF by pipetting samples carefully up and down up ten times, followed by 10 minutes incubation on ice. Samples were centrifuged at 14 000 rpm for 10 minutes at 4 °C and then directly mixed with 4 µl loading buffer. Mitochondria were applied on a native gradient gel, usually with an acrylamide gradient of 4-13 % at 1.5 mm spacer size in a Mini protean III chamber. Coomassie cathode buffer and anode buffer were carefully poured into the gel chamber, and samples were allowed to run into the gel at 100 V for 30 minutes at 4 °C. If western blot was planned afterwards, the cathode buffer containing Coomassie blue was replaced by a clear cathode buffer, and the run was completed at 180 V. For visualization of the native protein marker, the gel was stained with Coomassie R-250, and destained and fixed at the same time in Coomassie destaining solution.

2D-(BN-SDS-) PAGE

This method was adapted from Reisinger *et al.* [07]. Mitochondrial samples were subjected to BN-PAGE as on 4-10 % BN-gels. Then, gel strips of single lanes were cut out and denatured for 30 minutes in 1 % SDS and 1 % β-mercaptoethanol. Strips were rinsed with ddH_2O for 30-60 seconds to remove β-mercaptoethanol. Then, they were placed between clean glass plates from a PegLab 10 x 14 cm chamber with a thinner spacer then used during the first dimension. An SDS resolution gel with 12.5 % acrylamide was carefully poured underneath the strip and overlayed with ddH_2O until polymerized. The stacking gel was poured above the running gel until it embedded the first dimension while care was taken that no air bubbles arise between the layers. The gel was run in 1x SDS running buffer.

Western blot

For immunoblot, the semi-dry method was used. The BN- or SDS-gel was shortly rinsed with 1x semi-dry buffer and was placed on a PVDF-membrane that had been activated with methanol and equilibrated in 1x semi-dry buffer. Two 3 mm Whatman papers soaked with 1x semi-dry buffer were placed below and on top of the gel-membrane sandwich. The transfer of proteins was carried out at 100 mA for 1.5 h. If the membrane was still blue after blotting of BN-gels the membrane was destained with methanol and Coomassie destaining solution followed by intense washing in PBS-T. After the transfer membranes were blocked in 10% milk powder and 3% BSA in TBS-T for 1 hour at room temperature while gently shaking. When immunodecoration with an antibody was planned that gave a high background or when the transferred proteins had been separated on a native gel, blocking was performed over night at 4 °C. After briefly rinsing the membrane with TBS-T, it was overlayed with a primary antibody diluted in 3 % BSA in TBS-T and shaken at room temperature for 2 hours or over night at 4 °C, depending on the quality of the antibody. The membrane was washed for 30 to 45 minutes with TBS-T on a shaker under frequent exchange of the washing solution. Then, a secondary antibody coupled to horse radish peroxidase was applied for 45-60 minutes at room temperature. Following thorough washing with TBS-T, antibody binding was detected by activating the peroxidase with ECL substrate. Chemoluminescence was detected by Hyperfilm-ECL films.

9.4 DNA methods

Cloning of shRNA-encoding DNA into pLV-THM

ShRNA-encoding DNA was ordered for both sense and antisense strand as oligonucleotides and annealed in a gradient thermocycler. The pLV-THM vector was cut with MluI and ClaI, followed by trimming with T4-DNA polymerase in the presence of dGTP. The processed vector was ligated with the annealed oligonucleotides.

Oligo annealing	Vector digestion	T4 reaction	Ligation
10 µM sense oligo	20 µg vector	30 µl vector DNA	100 ng vector DNA
10 µM antisense oligo	20 U Cla1	2 mM dGTP	2.5 µl oligos
1x annealing buffer	20 U Mlu1	1x NEBuffer2 (10x)	1x ligase buffer (10x)
→ filled up to 50 µl	1x NEBuffer 4 (10x)	1x BSA (100x)	20 U T4 ligase
with ddH_2O	1x BSA (100x)	6 U T4 DNA polymerase	→ filled up to 20 µl
	→ filled up to 100 µl	→ filled up to 40 µl	with ddH_2O
	with ddH_2O	with ddH_2O	
Incubation:	Incubation:	The reaction was	Incubation:
4 minutes at 94 °C	over night at	pipetted on ice.	Over night at 4 °C
10 minutes at 70 °C	room temperature.	Incubation:	
70 – 4 °C (-0.1 °C /sec)	Purification with the	30 minutes at 12 °C,	
	PCR purification kit.	10 minutes at 75 °C	

The ligated vector was transformed into *E. coli* DH5α and plated on LB-medium with 10 µg/ml ampicillin. Single colonies were picked and screened for the presence of insert by colony PCR. One positive clone was sequenced by MWG.-Biotech AG.

Cloning of *in vitro* expression vectors

For cloning the *in vitro* expression vectors, pGEM-4z was used as a backbone. Bak and Bax genes were amplified from the vectors pEGFP-C2 Bak and pEGFP-C2 Bax by PCR. PCR products and backbone vector were digested with HindIII and EcoRI. Ligation reactions were transformed into competent *E. coli* DH5α, and positive clones were selected by colony PCR and sequencing.

PCR	Digestion	Annealing
10 ng template DNA	3 µg vector or 1 µg PCR product	150 ng vector DNA
200 µM each dNTP	5 U HindIII	300 µg PCR product
0.5 µM each primer	5 U EcoRI	1x ligase buffer (10x)
1x Phusion buffer	1x NEBuffer EcoRI (10x)	20 U T4 ligase
1 U Phusion polymerase	→ filled up to 50 µl with ddH_2O	→ filled up to 20 µl
→ filled up to 40 µl with ddH_2O		with ddH_2O
Incubation:	Incubation:	Incubation:
1 minute at 98 °C,	3.5 hours at 37 °C	2 hours at
10 seconds at 98 °C |	20 minutes at 65 °C	room temperature.
30 seconds at 72 °C | 30 cycles	Purification with the PCR	
20 seconds at 66 °C |	purification kit.	
10 minutes at 72 °C		
Purification with the PCR purification kit.		

pGEM-4z-Bax S184V was obtained by whole vector amplification of pGEM-4z-Bax with phosphorylated primers encoding the point mutation. Purified PCR products were processed with T4 DNA polymerase. The resulting linear vector was ligated with 10 U T4-ligase over night at 16 °C and digested with DpnI to delete methylated template DNA. Vector DNA was transformed into competent E. coli DH5α, and a positive clone was selected by sequencing.

PCR	T4 reaction	DpnI digestion
10 ng template DNA	3 µg vector DNA from PCR	10 µl ligation reaction
200 µM each dNTP	2 mM dGTP	10 U DpnI
0.5 µM each primer	1x NEBuffer 2 (10x)	1x NEBuffer4 (10x)
1x buffer 2	1x BSA (100x)	→ filled up to 20 µl
1.5 U Expand High Fidelity ^{plus} polymerase	3 U T4 DNA polymerase	with ddH_2O
→ filled up to 50 µl with ddH_2O	→ filled up to 40 µl with ddH_2O	Incubation:
Incubation:	The reaction was pipetted on ice.	30 minutes at 37 °C
2 minutes at 94 °C	Incubation:	
30 seconds at 94 °C |	20 minutes at 65 °C	
30 seconds at 52 °C | 30 cycles	Purification with the PCR	
3.3 minutes at 68 °C |	purification kit.	
10 minutes at 68 °C		
Purification with the PCR purification kit.		

Heat shock transformation

100 µl competent *E. coli* DH5α were mixed with DNA and incubated on ice for 20 minutes. The heat shock was applied for 45 seconds at 45 °C, followed by 2 minutes on ice. Bacteria were then diluted in 4 volumes of prewarmed LB-medium and shaked at 37 °C for 45 minutes. Then bacteria are spinned down at 5000 g for 2 minutes and plated on LB-Agar with the respective antibiotica for selection. Bacteria are grown over night at 37 °C under 5 % CO_2.

Colony PCR

To screen for positive colonies after plasmid cloning, 1 colony was picked, streaked on a LB-plate and dissolved in 25 µl PCR mix (1x Taq-buffer, 0.25 mM each dNTP, 500 µM each primer, 0.25 µl Taq-Polymerase). Initial denaturation was carried out for 2 minutes at 95 °C, then 25 cycles of 30 seconds at 94 °C, 30 seconds at the appropriate annealing temperature and 1 minute / kb at 68 °C were performed, followed by a final elongation step of 5 minutes at 68 °C. PCR products were controled on 1 % agarose gels.

Quantitative real-time PCR

RNA was isolated from cultured cells using the Qiagen Rneasy Mini Kit according to the manufacturer´s protocol. The sample concentration was determined and diluted to 1 ng/µl with RNAse-free water. Reverse transcription and quantitative real-time PCR (qRT-PCR) were carried out in one step in a 96-well microtiter plate. Each sample was subjected to qRT-PCR in triplets using GAPDH levels as a standard. Results were analyzed with the SDS2.2.2 program, knockdown levels were calculated according to the model by Pfaffl [Pfaffl, 01].

Reaction mix	Program	
10 ng RNA	30 minutes at 50 °C	
1x QuantiTect SYBR Green RT-PCR Master Mix (2x)	15 minutes at 95 °C	
0.4 µM each primer	20 seconds at 94 °C	45 cycles
0.25 µl QuantiTect RT Mix	40 seconds at 60 °C	
→ filled up to 25 µl with RNAse free ddH_2O	40 seconds at 72 °C	
	15 seconds at 95 °C	
	15 seconds at 60 °C	
	15 seconds at 95 °C at ramp rate of 2 %.	

9.5 Microscopy

Immunofluorescence

For immunoflourescence assays, cells were seeded onto glass coverslips. In some experiments, Mito-Tracker Orange staining prior to fixation was performed. Therefor, 150 nM MitoTracker Orange was diluted in prewarmed medium and incubated on living cells for 30 minutes at 37 °C in the dark. For fixation, cells were washed once with PBS and incubated with 3.4 % paraformaldehyde for 30 minutes at room temperature. After washing in PBS, cells were blocked and permeabilized in blocking buffer containing 10 % goat serum and 0.2 % Triton-X for 30 minutes at room temperature. Cells were washed with PBS and incubated with primary antibody, diluted 1:100 in antibody dilution solution for 1 hour at room temperature. Following three washing steps with PBS, samples were blocked a second time in blocking buffer for 10 minutes at room temperature. Samples were then rinsed with PBS and probed with a secondary antibody labeled with a fluorchrome that was spinned down at 10 000 rpm for 2 minutes before diluted 1:100 in antibody dilution solution. Antibody incubation for 1 hour in the dark at room temperature was followed by two washing steps with PBS and one washing with ddH_2O. The coverslips were then placed upside down on a drop of moviol on glass slides and dried in the dark. Samples were stored at 4 °C until analyzed by a confocal microscope.

Transmission electron microscopy

Transmission electron microscopy was performed by the microscopy core facility of the Max-Planck-Institute. Cells were fixed with 2.5 % glutaraldehyde and resin embedded as described previously [Goosmann, 08]. In brief, cells were contrasted using osmium tetroxide, tannic acid and uranyl acetate and dehydrated in a graded ethanol series. The monolayer was harvested with styrol and embedded in Polybed epoxi resin. Ultrathin sections were cut on an ultramicrotome, lead citrate contrasted in a TEM stainer and analyzed in a LEO 906E transmission EM with a side-mounted digital camera. Mitochondrial size was determined by measuring areas using ImageJ software.

List of abbreviations

3´UTR	three prime untranslated region
AGO2	Argonaute 2
APS	ammonium persulfate
ATP	adenosine triphosphate
BH	Bcl-2 homology
BN-PAGE	blue native-polyacrylamide electrophoresis
bp	base pair
C-terminal	carboxy-terminal
cyt *c*	cytochrome *c*
DIABLO	direct IAP binding protein with low PI
DISC	death-induced signaling complex
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	desoxyribonucleotid triphosphate
Dox	doxycycline
ds	double stranded
dsRed	*Discosoma* red fluorescent protein
E. coli	*Escherichia coli*
F1α	alpha subunit of the ATPase F1 complex
FACS	fluorescence activated cell sorting
FADD	Fas-associated death domain
GFP	green fluorescent protein
GTP	guanosine triphosphate
HEPES	2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonsäure
IAP	inhibitor of apoptosis
IMM	inner mitochondrial membrane
IMP	inner membrane peptidase
IMS	intermembrane space
IRES	interal ribosome entry site
kDa	kilodalton
KRAB	Krüppel-associated box
LTR	long terminal repeat
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MIA	mitochondrial intermembrane space import and assembly
miRNA	microRNA
MMP	mitochondrial processing peptidase
MOI	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
mtDNA	mitochondrial DNA
Mtx1	Metaxin 1
Mtx2	Metaxin 2
N. crassa	*Neurospora crassa*

N. gonorrhoeae	*Neisseria gonorrhoeae*
N. meningitidis	*Neisseria meningitidis*
nt	nucleotide
N-terminal	amino-terminal
OD	optic density
OMM	outer mitochondrial membrane
ORF	open reading frame
P.IA	porin B isoform IA
PAM	precursor-associated motor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PK	proteinase K
Pol III	polymerase III
PorB	porin B
qRT-PCR	quantitative real-time PCR
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rotations per minute
S. cervisiae	*Saccharomyces cervisiae*
SAM	sorting and assembly machinery
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide electrophoresis
shRNA	short hairpin RNA
SIN	self-inactivating
siRNA	small interfering RNA
ss	single stranded
SW	swelling
tBid	truncated Bid
TCA	trichloric acid
TEM	transmission electron microscope
TEMED	*N*, *N* ,*N´*,*N´*-Tetramethylethan-1,2-diamin
tetO	tet Operon
TIM	translocase of the inner mitochondrial membrane
TMS	transmembrane segment
TNFα	tumor necrosis factor alpha
TOM	translocase of the outer mitochondrial membrane
TRADD	TNFR associated death domain
Tris	tris(hydroxymethyl)aminomethane
U	unit
VDAC	voltage-dependent anion channel
w/v	weight per volume
WB	western blot
Δψ	mitochondrial inner membrane potential

SI units, SI derived units and SI prefixes were used. Amino acids and chemicals were abbreviated according to the IUPAC nomenclature.

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11 Appendix

Publications

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Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur mit den angegebenen Hilfsmitteln erstellt habe.

Berlin, März 2009

Katharina Ross